



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C07K 16/40, C12N 9/78, A61K 39/395, 38/50, 51/08, G01N 33/573, 33/574, 33/577, C12N 5/20</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/12618</b>  <b>(43) International Publication Date:</b> 11 May 1995 (11.05.95)
<b>(21) International Application Number:</b> PCT/EP94/03632  <b>(22) International Filing Date:</b> 4 November 1994 (04.11.94)  <b>(30) Priority Data:</b> 93203093.5 4 November 1993 (04.11.93) EP <b>(34) Countries for which the regional or international application was filed:</b> AT et al. 9400309 28 February 1994 (28.02.94) NL  <b>(71) Applicants (for all designated States except US):</b> EURO-GENETICS N.V. [BE/BE]; Transportstraat 4, B-3980 Tessenderlo (BE). UNIVERSITAIRE INSTELLING ANTWERPEN [BE/BE]; Universiteitsplein 1, B-2610 Wilrijk (BE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DE MEESTER, Ingrid [BE/BE]; Laaglandweg 71, B-2610 Wilrijk (BE). VAN HAM, Guido [BE/BE]; Plashoevestraat 7, B-2500 Lier (BE). KESTENS, Luc [BE/BE]; Beschavingstraat 6, B-2020 Antwerpen 2 (BE). VANHOOF, Greta [BE/BE]; Deurnestraat 269, B-2640 Mortsel (BE). VERHELST, Peggy [BE/BE]; Van Luppenstraat 47, B-2018 Antwerpen (BE). GOOSSENS, Filip [BE/BE]; Hoogstraat 91, B-9160		Lokeren (BE). HENDRIKS, Dirk [BE/BE]; Zwaluwenlaan 9, B-2630 Aartselaar (BE). BORLOO, Marianne [BE/BE]; Van Notenstraat 142, B-2100 Deurne (BE). HAEMERS, Achiel [BE/BE]; Voskenslaan 399, B-9000 Gent (BE). BOSMANS, Eugène [BE/BE]; Beverzakkbroekweg 91, B-3520 Zonhoven (BE). SCHARPE, Simon [BE/BE]; Kerkhofstraat 7, B-9280 Wieze (BE).  <b>(74) Agent:</b> VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).  <b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A LIGAND CAPABLE OF BINDING TO THE ADENOSINE DEAMINASE BINDING-SITE OF CD26  <b>(57) Abstract</b>  <p>The invention relates to a ligand or functional derivative thereof, with at least one specificity for the adenosine deaminase (ADA) binding site of CD26, being for example a monoclonal antibody or a modified version of adenosine deaminase. The functional derivative can be an F<sub>ab</sub>-, F<sub>ab</sub>'-, F<sub>(ab)2</sub>-, an scF<sub>v</sub>- or F<sub>v</sub>-fragment of the monoclonal antibody or a chimeric antibody. Apart from this the functional derivative can be a conjugate between the ligand or a functional component thereof and a secondary molecule. The ligands according to the invention can be used as a therapeutic agent for stimulating the proliferation of T-lymphocytes, as an immuno-suppressing agent, as a diagnostic agent in immuno-enzymatic assays, for monitoring malignantly transformed melanocytes or in imaging techniques or as therapeutic agent against HIV-infection.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

**A LIGAND CAPABLE OF BINDING TO THE ADENOSINE  
DEAMINASE BINDING-SITE OF CD26**

The invention relates to ligands such as modified  
5 versions of adenosine deaminase (ADA) for instance, or a  
monoclonal antibody or functional derivative thereof,  
capable of binding to the adenosine deaminase binding-site  
of CD26. Furthermore, the invention concerns the use of the  
ligands in various therapeutic and diagnostic applications  
10 and a hybridoma cell line which produces an antibody  
according to the invention.

Apart from the classic T-cell receptor-CD3-complex  
(TcR-CD3), different other molecules appear to be involved  
in co-stimulation of the lymphocytes. CD26 is such a plasma  
15 membrane molecule. It is an activation marker, which means  
that its expression is increased on active T-cells.

The enzyme adenosine deaminase (ADA) catalyses the  
irreversible deamination of adenosine and 2' deoxy-adenosine  
to inosine and 2'deoxyinosine, respectively. An hereditary  
20 shortage of ADA-activity causes a serious weakening in the  
cellular and humoral immunity (1). It is thought that the  
accumulation of toxic substrates and metabolites interferes  
with the T-cell activity and  
-proliferation. The mechanism is, however, not clear at  
25 present.

ADA is widely spread in mammalian tissue where the  
highest levels are found in the thymus and other lymphoid  
tissues (2, 3). Genetic polymorphism and post translational  
modifications generate different physiological forms of ADA  
30 (2, 4, 5). In humans, the ADA-activity occurs substantially  
in two groups of iso-enzymes ADA-S and ADA-L (6). ADA-S is  
found in erythrocytes and consists of a single polypeptide  
with a molecular weight of between 36 and 44 kDa. The ADA-L-  
group of iso-enzymes, which are also called "tissue iso-  
35 enzymes" has a higher molecular weight and results from the  
interaction of the ADA-catalytic unit with a homodimeric  
glycoprotein of roughly 200 kDa. This glycoprotein, which  
is substantially membrane-bound, is called ADA binding

- 2 -

protein (ADAbp) or ADA complexing protein (ADAcP). A native molecule of ADAbp binds two molecules of catalytic ADA. Up until now, no physiological role for ADAbp (7) has been determined. Dinjens et al. have shown that the membrane-bound ADAbp is not completely saturated with ADA-S. They suggest that due to its capability to bind ADA-S, the membrane bound ADAbp would regulate the circulation level of adenosine deaminase activity (7).

It has recently been shown that ADAbp from the human kidney is identical or analogous to the very specific ecto-enzyme dipeptidyl peptidase IV (DPP IV) (8). The molecular and biochemical characteristics and the tissue distribution of ADAbp and DPP IV appear to be inseparable (7, 9). Within the hematopoietic system, the DPP IV is, apart from this, identified as the membrane protein CD26 (10) and is substantially expressed on a subpopulation of T-cells.

It is known that, amongst other things, CD26 is related to HIV-infection (25). Furthermore the disappearance of CD26 expression is reproduced on malignant transformed melanocytes. Because CD26 is an activation marker, the CD26 expression is also a measure for determining the extent of cell activation.

It has now been found that ligands, which bind to CD26, such as ADA itself or modified versions thereof for example, and antibodies or derivatives thereof, can have an influence on the activity or expression of CD26 and can also form a diagnostic agent for determining the CD26 expression.

Hence, for various diagnostic and therapeutic applications the provision of a ligand binding to CD26, such as for example a specific anti-CD26 antibody, is especially desirable.

The present inventors, in order to achieve this, have produced amongst other things, a monoclonal antibody which is directed against the ADA binding site of CD26. The present invention is directed to every conceivable ligand that is directed to the ADA binding site of CD26. These ligands, for example antibodies, may be prepared and selected by methods well known to the skilled person. One

- 3 -

example of such a ligand is the monoclonal antibody anti-TA5.9, which is produced, for example, by hybridoma cell line anti-CD26-(TA5.9-CC1-4C8) which was deposited on 4 November 1993 at the European Collection of Animal Cell Cultures, Salisbury, GB, under the deposit accession number ECACC 93110419.

It has specifically been found that the anti-TA5.9 antibody according to the invention has various effects. However, it has to be understood that this specific antibody is only one embodiment of the invention illustrating its inventive concept.

One of the effects is that a strong co-stimulation of T-lymphocytes activity via CD3 takes place in vitro. This applies for both CD4+ (helper) and the CD8+ (cytolytic) T-cells. It is a prerequisite that the cells express CD26 on their membrane.

In the absence of soluble or immobilized anti-TA5.9 monoclonal antibody, a downmodulation of CD26 expression occurs. This downmodulation is important for the co-stimulation via CD26.

However, the membrane protein CD26 also functions as a co-receptor for the HIV-virus. Downmodulation of CD26 via anti-TA5.9 can appear to be useful here in order to prevent entrance of the virus into the target cell in vitro or in vivo.

The anti-CD26 monoclonal antibodies, with specificity for the 5.9 epitope, cause an immunosuppression which is specific for the CD26+ subset of T-cells in vivo. Such an immunosuppression can be important after transplantations and with autoimmune diseases such as multiple sclerosis, rheumatism, SLE etc.

Another application of anti-TA5.9 according to the invention is found in diagnosis. Malignant transformed melanocytes lose the CD26 expression on their cell membrane. By means of anti-TA5.9 it is possible to trace the disappearance of CD26 and determine the number of malignant transformed melanocytes therewith.

Anti-TA5.9 may also be used in diverse immuno-enzymatic determinations. The soluble homodimeric CD26 can be determined in an ELISA. It is also possible to detect the CD26 by means of its own enzymatic activity. Serum, plasma, 5 seminal fluid and tissue preparations can be used as samples.

Anti-TA5.9 can be employed for determining ADA in biological fluids due to its ADA binding-site recognition characteristics.

10 When anti-TA5.9 is labelled with an isotope or magnetic resonance label for example, it can be used in imaging-techniques, such as scintigraphy or MRI (Magnetic Resonance Imaging). CD26 expression can be recognized in the body or in tissues with such techniques.

15 The above mentioned effects of the anti-TA5.9 can of course also be achieved with other ligands binding CD26 such as ADA itself or modified versions thereof. By modification one thinks of for example coupling to a secondary molecule selected from the group which consists of 20 toxins, enzymes, medicines, radio active markers, fluorescent markers, magnetic resonance markers, proteases, synthetic and natural polymers.

The invention comprises apart from ADA and anti-TA5.9, furthermore all the conceivable functional 25 derivatives thereof. By "functional derivatives" is to be understood each of the molecules derived from the ligand comprising at least one binding-site with a specificity for the adenosine deaminase (ADA) binding-site of CD26. Such a derivative may be produced by means of recombinant DNA

30 techniques, for example, or in any other suitable way. Examples of the derivatives of the monoclonal antibody are antibody fragments such as the  $F_{ab}$ -,  $F_{ab}$ '- or  $F_{(ab)2}$ - fragment which consists of the antigen binding parts of an immunoglobulin molecule, or the  $F_v$ - or  $scF_v$ -fragment, 35 comprising the variable range of the immunoglobulin. The term " $scF_v$ " stands for the so called "single chain" antibody which consists of the variable parts of the heavy and light chains of the immunoglobulin molecule, which are coupled to

each other by means of a linker peptide which may or may not be synthetic.

By the term "functional derivatives" are also understood the combination of at least the parts of the  
5 ligand required for binding to the ADA binding-site of CD26 (named the "functional components") with at least one other molecule such as a toxin, enzyme, marker, transport protein, synthetic or natural polymer, medicines etc.

Furthermore the term "functional derivatives"  
10 comprises chimeric antibodies, such as humanized antibodies and also by specific antibodies. The latter have two different antigen binding-sites and thereby specificities. At least one specificity is always directed towards the adenosine deaminase binding-site of CD26 while the other  
15 specificity can be directed towards any desired antigen. Here is understood other plasma membrane proteins such as CD12, CD3, CD8, CD4, markers, such as radioactive, fluorescent or magnetic resonance markers but also medicines, toxins and all other conceivable molecules which  
20 would be able to be functional in combination with CD26.

The ligands according to the invention can find a therapeutic application in the so-called "targeting". Targeting is used for bringing effector cells, medicines, toxins, imaging markers, natural and synthetic polymers etc.  
25 for example to the CD26+ cells.

A bispecific antibody which apart from the specificity for CD26 also contains a specificity for CD12, a marker for Natural Killer (NK) cells, will bring NK-activity to the target cell (CD26+). The adapted combination of CD26  
30 and CD3 can cause proliferation of T-cells. Bispecific antibodies directed against both CD26 and CD8 can induce cytolysis of the cell concerned while due to the specificity for CD26 and CD4, the attachment and entry of HIV in target cells can be prevented.

35 The common characteristic of the ligands according to the invention and all the functional derivatives thereof is the specificity for the ADA binding-site of CD26. In the

claims under the term "ligand" is also understood all the functional derivatives thereof.

The invention shall further be exemplified by reference to the following examples which are not however  
5 meant to limit the invention in any way.

#### **EXAMPLE 1**

##### ADA-binding on the lymphocyte surface

###### 10 1. Introduction

Some monoclonal antibodies which are specific for CD26, exhibit co-stimulating activity for TcR/CD3 induced T-cell activity (11, 12). De DPP-IV-activity of CD26 plays an important role, not only in CD26 mediated co-stimulation,  
15 but also in IL-2-production after different stimuli (13-15). The expression of CD26 on the surface (16) and the specific DPP-IV-activity (17) is increased after T-cell-activation. The functional importance of the CD26 antigen and its DPP-IV-activity in the T-cell-activation cascade, together with  
20 the apparently essential role of ADA in the development of normal immune responses, has brought the inventors to examine different aspects of the interaction between ADA and the lymphocytic CD26/DPP-IV. In this way the binding of the monoclonal antibody anti-TA5.9 according to the invention,  
25 which is directed against CD26, and a few other anti-CD26 monoclonals, to different lymphocyte subsets has been compared with the surface expression patterns of ADABp. Apart from this, the influence of ADA on the interaction between CD26 and the monoclonal antibodies, which recognize  
30 differing epitopes on the CD26, were studied. Furthermore, an analysis was carried out as to the effect of ADA on DPP-IV-enzyme activity. Moreover, the direct binding of pure CD26/DPP IV to immobilized ADA was proved. Finally the influence of ADA on CD26 surface expression was analyzed.



## 2. Materials and methods

### 2.1 The preparation of monoclonal antibody anti-TA5.9.

The monoclonal antibody anti-TA5.9 is produced by the hybridoma cell line anti-CD26-(TA5.9-CC1-4C8), which on 5 November 4, 1993 was deposited at the European Collection of Animal Cell Cultures, Salisbury, GB, under the deposit accession number ECACC 93110419. The hybridoma cell line, here after referred to with the name LY12.CC1, was obtained in the manner described below. Information about these known 10 techniques may be found in J. Eryl Liddell & A. Cryer, "A practical guide to monoclonal antibodies", John Wiley & Sons (1991), for example.

The immunization took place by the intraperitoneal injection of BALB/c-mice with PHA stimulated human 15 peripheral blood mononuclear cells. Complete cells were used suspended in PBS, without the addition of an adjuvant.

The hybridomas were produced by fusing spleen cells of the immunized mice with SP2/O-AG14 myeloma cells using PEG as the fusing promotor. The hybridomas were 20 selected by growing them on HAT-medium.

Thereafter the hybridomas were screened on the production of antibodies which were specific for human mononuclear cells activated with PHA. All the screening tests were carried out by means of flow-cytometry. In the 25 first screening test, the antibody reactivity of the cells stimulated with PHA was measured. Antibodies which exhibited a strong reactivity in this first screening were again tested in the second screening test for binding to resting human peripheral blood mononuclear cells. Antibodies which 30 exhibited a significantly higher reactivity with the PHA stimulated cells in comparison with resting cells, were selected for further development.

The selected hybridoma CC1 was twice cloned by limited dilution. Microtiter wells which contained a single 35 growing cell colony were retested in the same screening procedure. Clone LY12.CC1 was chosen for further proliferation and antibody production. The antibodies were produced in the ascites liquid of the BALB/c-mice which were

injected with cells from the LY12.CC1 clone. By means of affinity chromatography on protein A Sepharose, monoclonal antibody was purified from the ascites fluid.

The binding characteristics of the monoclonal  
5 antibody LY12.CC1 with different types of cells, was tested by flowcytometry. The antibody exhibited a high reactivity with all the tested preparations of human peripheral blood mononuclear cells stimulated with PHA. Up to 98% of these cells reacted positively. On the contrary, resting human  
10 peripheral blood mononuclear cells generally exhibited a weaker reactivity with this antibody, while a clear differentiation between the populations of a positive of negative reacting cells was detected. Furthermore, simultaneous two color cytometric analysis showed that the  
15 positive reacting cell population of the resting mononuclear cells, consisted of a subpopulation of T-cells including CD4 positive and CD8 positive T-cells. Similar studies showed that this antibody did not react with the T-cell activation markers CD25 (IL2-receptor), CD71 (transferrin receptor) or  
20 HLA-DR. Subtyping disclosed that the antibody was a mouse IgG1 monoclonal antibody.

## 2.2 Cell preparation and FCM-analysis

Human Peripheral Blood Mononuclear Cells (PBMC)  
25 were isolated from blood made oncoagulatable with EDTA, by density gradient-centrifugation on Ficoll-Paque, such as described earlier (18). The PBMC were harvested for 4 days on a density of  $0,5 \times 10^6/\text{ml}$  in RPMI-1640-medium supplemented with 10% FCS, glutamine and antibiotic (further called  
30 "complete medium") in the presence of  $0.5 \mu\text{g}/\text{ml}$  PHA (phytohemagglutinin, purified quality from Wellcome, Dartford, England).

Indirect immunofluorescence-staining of washed PBMC, which were harvested or originated from complete  
35 blood, was carried out in PBS with 1% BSA, 0.05% sodium azide, using the anti-CD26 antibodies anti-Ta1 (Coulter, Hialeach), anti-BT5.9 (obtained from Dr. Corte, Genua, Italy) and the anti-TA5.9 according to the invention. Apart

from this, the anti-ADABp-monoclonal URO-4 (clone S-27) from Signet Laboratories, Dedham, MA, was used. After incubation of the specific monoclonal antibodies for 20 minutes at 4°C, the cells were twice washed and the F(ab')<sub>2</sub> (produced in  
5 goat en directed against mouse IgG) that was supplied with FITC or PE (Tago, Burlingham, Ca) was added during an other 20 minutes at 4°C. In the case of complete blood samples, the red blood cells were lysated before washing and fixating of the cells. The samples were analyzed on a FACScan flow  
10 cytometer (Becton Dickinson, Erembodegem, Belgium). For direct immunofluorescence, FITC and PE conjugated in monoclonal antibodies from Becton Dickinson (apart from anti-TA5.9-FITC) were used. The samples were incubated and lysated for 20 minutes at 4°C, washed and fixed as described  
15 above. As a control, iso-type monoclonal antibodies with a known specificity were used.

Two color experiments with one non-conjugated and one conjugated specific monoclonal antibody were carried out as follows. The samples were incubated (20 minutes, 4°C)  
20 with a non-conjugated monoclonal antibody and washed. F<sub>(ab')</sub> (produced in goat directed against mouse-IgG) labeled with FITC or PE was added thereto during a further 20 minutes at 4°C. After washing the free binding-sites on cell bound goat-anti-mouse-fragments were blocked with 5 µl of mouse  
25 serum. Finally the directly conjugated monoclonal antibody was added for a last incubation and washing cycle. The samples were treated as described above.

### 2.3 Influence of ADA on FCM-analysis and expression of 30 lymphocytic CD26/DPP IV.

The influence of ADA on the binding of anti-CD26 monoclonal antibodies on the lymphocyte surface was tested by pre-incubation of stimulated PBMC or washed complete blood with differing amounts of bovine adenosine deaminase  
35 (type V, Sigma, Bornem, Belgium) for 30 minutes at room temperature in PBS with 1% BSA and 0.05% sodium azide. Thereafter the cells were twice washed with the same buffer.

Following this, indirect or direct staining of the cells was carried out as described above.

In order to study the downregulation of CD26 expression on the surface by ADA, the PBMC were freed from  
5 contaminating monocytes (by attachment to polystyrene plates) and incubated in complete medium at 37°C for different periods of time (2, 4, 8 and 24 hours) in the presence or absence of bovine-ADA (0.5 to 50 µg/ml) or anti-TA5.9 according to the invention (0.5 to 50 µg/ml). In a  
10 first series of experiments, ADA and the anti-TA5.9 monoclonal antibody were added in solution, and in a second series they were immobilized on polystyrene plates. After incubation, the samples were cooled on ice and indirectly stained with anti-Ta1 monoclonal antibody as described  
15 above.

#### 2.4. Interaction of ADA with purified CD26 dipeptidyl peptidase IV.

In order to study the influence of ADA on the  
20 enzymatic activity of DPP IV, bovine-ADA (0.02-1.7 µg/ml agree with 2-180 mU/ml) was incubated for one hour at 37°C with purified human dipeptidyl peptidase IV. Thereafter the DPP IV-activity was measured with using the fluorogenic substrate Gly-Pro-4-Methoxy-2-Naphtylamide such as described  
25 elsewhere (19). In order to study the effect of ADA on the interaction between purified DPP IV and immobilized monoclonal antibodies (20), anti-CD26 monoclonal antibodies (anti-Ta1, anti-TA5.9 according to the invention, anti-BT5.9, 134-2C2 and anti-1F7) were spotted directly onto a  
30 strip of nitrocellulose. The 134-2C2 and anti-1F7 monoclonal antibodies were obtained from Dr. Plana, Barcelona, Spain and Dr. Morimoto, Boston, MA resp. After blocking and washing such as described in (20) the monoclonal antibody containing strips were incubated at 4°C with DPP IV which  
35 had been previously incubated with bovine ADA (1 hour at 37°C). In blanc and control experiments, the strips were incubated in PBS-buffer or in DPP IV without ADA, respectively. After incubation overnight, the membranes were

washed twice with 20 mmole/l Tris-HCl, pH 7.4 with 1 mole/l NaCl and the DPP-IV bound was fluorometrically measured as described in (20).

The interaction between ADA and purified  
5 dipeptidyl peptidase IV was further investigated using affinity chromatography. ADA from calf intestines (type VI, Sigma) was coupled to CNBr-Sepharose 6B (Sigma) in a concentration of 5 mg/ml gel, as described by the supplier. The columns were equilibrated with 20 mmole/l Tris-HCl, pH  
10 7.4 with 0.5 mole/l NaCl in order to avoid a-specific binding. Preparations of purified DPP IV (100 mU), alone or previously incubated (1 hour at room temperature) with an excess of mouse-IgG1 with a non relevant specificity, anti-Ta1 monoclonal antibody, URO-4 monoclonal antibody or anti-  
15 TA5.9 monoclonal antibody according to the invention, were mounted on to the ADA-Sepharose column. The binding of CD26/DPP IV was measured by determining the DPP IV activity in the non-bound fraction. In order to control a-specific interactions with the carrier, the same preparations were  
20 loaded on blocked CNB4-Sepharose 6B.

### 3. Results and discussion

3.1 The binding profiles of anti-ADAbp monoclonal antibodies and anti-CD26 monoclonal antibodies on lymphocyte subsets  
25 are analogous.

The monoclonal antibody URO-4, produced against a human cancer kidney cell line, recognizes the adenosine deaminase binding protein from human proximal tubular cells, fibroblasts and HepG2 cells (21, 22). In order to further  
30 examine the reported analogy or identity between ADAbp and CD26, the monoclonal antibody URO-4 was introduced into flow cytometric analyses of complete blood or PBMC. Both sources gave similar results. As demonstrated in figure 1, the reactivity patterns of URO-4 on different lymphocyte subsets  
35 (CD4+ and CD8+ lymphocytes and (not shown) CD19+ and CD16/56+ lymphocytes) were completely comparable to those obtained with anti-CD26 monoclonal antibodies anti-Ta1 and anti-TA5.9 according to the invention. With the antibody

anti-TA5.9, information about the CD26 expression on T-cell subsets was obtained. Roughly 50% of the CD5 positive cells appeared to express membrane-bound ADAbp (23). Simultaneous staining with URO-4 and anti-TA5.9 monoclonal antibodies 5 showed that both antibodies bind to the same cells (fig. 2). This indicates that ADAbp and CD26/DPP IV are identical.

3.2 ADA blocks the binding of anti-TA5.9, but not that of anti-Ta1, to CD26 on the surface of the lymphocytes.

10 Fig. 3 shows the influence of ADA on the fluorescent profile of anti-TA5.9 and anti-Ta1 monoclonal antibodies. ADA strongly inhibits the binding of anti-TA5.9 to the surface of both resting and stimulating lymphocytes. The binding of anti-Ta1 and URO-4 monoclonal antibodies was, 15 however, not reduced by ADA (results not shown). The results indicated that ADA binds to or near the TA5.9 epitope of CD26. The Ta1 epitope (of which it is reported that it reacts with the HIV-1 regulation protein TAT (24)) did not appear to be related with ADA binding to the lymphocyte 20 surface. Earlier observations that the binding of ADA to kidney ADAbp did not inhibit the immunoprecipitation of ADAbp by URO-4 (21) are in accordance with our experiments where no influence of ADA on the binding of URO-4 to lymphocyte CD26 was detected.

25

3.3 ADA strongly reduces the binding of purified DPP IV to immobilized anti-TA5.9, but not to anti-IF7 and anti-Ta1 anti-CD26 monoclonal antibodies.

Apart from the flowcytometric analyses, binding 30 studies were carried out on the soluble DPP IV and DPP IV/ADA-complexes and the immobilized monoclonal antibodies such as outlined out in Materials and Methods. Complex-forming with ADA destroyed the binding of DPP IV and anti-TA5.9 almost completely, whilst a slight or no effect was 35 seen on the binding to anti-Ta1 and anti-IF7 monoclonal antibodies (data not shown). These results confirm that anti-TA5.9 is specific for the epitope on CD26, which is related to ADA-binding.

3.4 Anti-TA5.9 inhibits the binding of purified CD26/DPP IV to immobilized ADA.

The affinity chromatography experiments directly showed the interaction of CD26/DPP IV with ADA and the relation of the TA5.9-epitope in the binding between these two molecules. When CD26/DPP IV was alone loaded on an ADA-Sepharose 6B, 100% of the enzyme was binding to the column. CD26/DDP IV preparations which were incubated with anti-Ta1 or URO-4 monoclonal antibodies, were both binding for more than 85%. However, the anti-TA5.9 monoclonal antibody lowered the binding of CD26/DPP IV to immobilized ADA strongly to less than 20% of the quantity produced. This phenomenon was not caused by the a-specific binding of free anti-TA5.9 monoclonal antibody to ADA because in a control experiment 100% of the FITC labeled anti-TA5.9 monoclonal antibody on a ADA-Sepharose 6B column was found in the non bonded fraction.

3.5 ADA did not inhibit the DDP IV enzymatic activity.

In order to be confirm and extend the finding on the binding of ADA to CD26/DPP IV the effects of ADA on DPP IV enzymatic activity were studied. No decrease in Gly-Pro-4-methoxy-2-naphtylamide hydrolysis was found in DPP IV after incubation with varying concentrations of ADA (results not shown).

3.6 ADA does not modulate the CD26 surface

The ability of ADA to cause downregulation of CD26 expression on the cell surface, was invetsigated by analysis thereof after incubation of cells with varying concentrations of ADA for differing time intervals. Modulation of CD26 by anti-TA5.9 was carried out as a positive control. The results are shown in fig. 4.. After incubation with anti-TA5.9, the expression of CD26 on the surface began to decrease within two hours incubation time. High concentrations of anti-TA5.9 monoclonal antibody induced a quicker reduction in CD26-expression. ADA was not capable, under any of the given circumstances, to modulate

CD26-expression on the T-cell surface. An anti-Ta1 monoclonal antibody was used to detect CD26 on the cell surface, because the binding of ADA masks the TA5.9 epitope, but not the Ta1 epitope of CD26.

5           Until now, none of the possible ligands and/or substrates for CD26/DPP IV showed that they could induce the downregulation of CD26 surface expression. Anti-TA5.9 does this.

#### 10 4. Conclusion

The ecto-enzyme DPP IV, which is identical to lymphocytic CD26 is also the ADA binding protein of these cells. This is an intriguing observation considering the known co-stimulatory effects of anti-CD26 monoclonals and  
15 considering the serious immune damage caused by ADA deficiency. A critical site for ADA binding to CD26/DDP IV which is selectively related to the epitope, which is recognized by the monoclonal antibody according to the invention anti-TA5.9 was found. The results reproduced here  
20 further show that ADA, when bound to DDP IV, forms a type bifunctional enzymatic complex that may be active in the bloodstream and in tissues. Under the experimental conditions used, ADA was not capable of modulating CD26 on the lymphocyte surface. This was surprising considering that  
25 the anti-TA5.9 monoclonal antibody which reacted with a very closely related or identical epitope, quickly downregulated the CD26-expression.

#### 5. Description of the figures.

30           Figure 1 shows the binding of the anti-CD26 and anti-ADAbp monoclonal antibodies on CD4+ (left column) and CD8+ (right column) lymphocytes. Whole blood was indirectly stained with the anti-ADAbp monoclonal antibody URO-4 (at the top), or the anti-CD26 monoclonal antibody anti-Ta1 (in  
35 the middle) and anti-TA5.9 according to the invention (below). In all cases FITC-labeled  $F_{(ab')_2}$  produced in goat and directed against mouse-IgG was used as the secondary



reagent. Anti-CD4-PE (left column) or anti-CD8-PE (right column) was used as the second marker.

Figure 2 shows the co-expression of anti-CD26 and anti-ADAbp on lymphocytes. Whole blood was stained with  
5 either PE-labeled  $F_{(ab')_2}$  alone produced in goat and directed against mouse-IgG (left) or URO-4 and PE-labeled  $F_{(ab')_2}$  produced in goat and directed against mouse-IgG. In both cases anti-TA5.9 was used as the secondary reagent.

Figure 3 shows the influence of ADA on the binding  
10 of anti-TA5.9 (left) and anti-Ta1 (right) on the surface of resting PBL (above) or PHA-stimulated PBL (below).  $2 \times 10^5$  cells were incubated in the presence or absence of ADA ( $6 \mu\text{g/ml}$ ) for 30 minutes at room temperature. Thereafter, the binding of anti-TA5.9 and anti-Ta1 to the lymphocyte surface  
15 was examined by indirect FCM.

Figure 4 shows that anti-TA5.9, but not ADA, induces the downmodulation of CD26. PBL was incubated for the given time intervals (0, 2, 4 and 8 hours) in complete medium at  $37^\circ\text{C}$ , as such (above) in the presence of ADA ( $5 \mu\text{g/ml}$ ) or the absence of anti-TA5.9 ( $5 \mu\text{g/ml}$ ) (below).  
20 Thereafter CD26 was visualized on the cell surface by FCM and using the anti-Ta1 and PE-labeled  $F_{(ab')_2}$  produced in goat and directed against mouse-IgG.

## 25 **EXAMPLE 2**

### Diagnostic determinations

#### 1. Introduction

Hereinbelow are described two assays for the determination of CD26 in serum en seminal plasma. On the one  
30 hand that is a fluorometric assay based on the protease-activity of DPP IV and on the other hand it is a sandwich-ELISA. The first step in both tests is based on the capture of CD26 by the monoclonal antibody anti-TA5.9 according to the invention. The fluorometric assay is a functional  
35 determination measuring the proteolytic activity of the captured CD26 with a fluorogenic substrate. The ELISA determines the immunoreactive quantity of the soluble homodimer CD26.

## 2. Fluorometric assay for the determination of CD26.

### 2.1 Introduction

In the fluorometric assay, the activity of CD26 in serum or seminal fluid is measured. The CD26 is captured by the anti-TA5.9 applied to an adapted carrier. After washing away the unbound material, a fluorogenic substrate Gly-Pro-7-amino-4-methoxy coumarin is added and split by the bound CD26 in order to free a fluorescent molecule, in this case 7-amino-4-methoxy-coumarin. This last molecule can be directly measured (see fig. 5).

### 2.2 Method

Non-transparent microtiter plates of Nunc (Denmark), were covered with anti-TA5.9 (overnight at 4°C in a carbonate buffer, pH 9.6, 3 µg/ml). Remaining free binding sites, were blocked the following morning with 3% BSA in PBS pH 7.2. A pool of 10 different seminal fluids was made as a reference standard and dissolved in human albumin matrix (SOPP, Belgium Red Cross). The DPP IV activity of this reference standard was determined according to the method described in reference 19.

The reference standard was diluted in SOPP to 25 mU/ml, 2.5 mU/ml and 0.5 mU/ml. A series of dilutions was made from the seminal liquid from 100 x to 10.000 x.

100 µl of standard and test samples were placed in the wells of the microtiter plates. Incubations were carried out for two hours. A substrate stock solution of Glyco-Pro-7-amino-4-methoxy coumarin (20 mmol/l in DMSO) was diluted 12 times in incubation buffer (50 mM Tris-HCl, pH 8.3).

Thereafter the plates were washed 5 times with PBS pH 7.2 buffer with Tween 80. Following this, 100 µl of the diluted substrate solution was added to each well. Incubation was then carried out for one hour at 37°C. The reaction was stopped by the addition of 100 µl 1M citrate pH 4.0 to each well. The intensity of the fluorescence signal was determined in a Fluoskan instrument at 355 nm and 425 nm.

### 2.3. Results

The results of a population study, carried out on a series of 48 healthy blood donors with the fluorometric assay for CD26, are represented in fig. 5.

5

### 3. ELISA for the detection of CD26

#### 3.1. Introduction

Microtiter plates were coated with the anti-TA5.9 monoclonal antibody for capturing CD26 from the sample.

10 After washing away the non-bound material, the bound homodimer CD26 was detected by biotinylated anti-TA5.9 monoclonal antibody and streptavidin-HRP. The HRP (horseradish peroxidase) reacted with  $H_2O_2$  and the chromogenic tetramethylbenzidine (TMB). This resulted in a  
15 blue reaction product which underwent a change of color to yellow after stopping the substrate reaction with 2M  $H_2SO_4$ . See fig. 6.

#### 3.2. Method

20 The reference standard (see under 2.2) was diluted in SOPP to 25 mU/ml, 2.5 mU/ml and 0.5 mU/ml. The seminal fluid, which was used as the test sample, was serially diluted from 100 x to 10.000 x. 100  $\mu$ l of standard- and test samples were placed in the wells and incubated for two hours  
25 at 37°C. Thereafter the plates were washed 5 x with a wash solution (PBS-buffer, pH 7.2 with 82). 100  $\mu$ l of the biotinylated conjugate was then added to each well. The biotinylated antibody was diluted in TBS-buffer, pH 7.2 with 0.1% Tween 20 and 2.5% BSA. The buffer in which the  
30 biotinylated monoclonal antibody is diluted, contains then 25  $\mu$ g/ml of mouse-IgG1. After an incubation of 1 hour at 37°C, the plates are washed 5 x with wash solution. Thereafter 100  $\mu$ l streptavidin-HRP is added to each well. The streptavidin is diluted in the same way as the  
35 biotinylated antibody. After an incubation of 20 minutes at 37°C, the plates are washed 5 x with wash solution, after which 100  $\mu$ l of  $H_2O_2$  substrate solution and 100  $\mu$ l of chromogenic solution (TMB, tetramethyl benzidine) is added

to each well. After an incubation of 20 minutes at 37°C the reaction is stopped by addition of 50 µl stopping buffer. Finally the absorption was determined at 450 nm.

### 5 3.3. Results

The results for 245 serum samples of healthy blood donors between 18 and 65 years, were analyzed. The results are shown in the graph of figure 7.

Apart from this 55 seminal plasma samples were 10 analyzed. The results are shown in the graph of fig. 8.

4. Enzyme-immunoassay (EIA) for determining adenosine deaminase in biological fluids.

### 15 4.1. Introduction

Due to the specific recognition of ADA-binding sites on CD26 by TA5.9, the monoclonal can, in combination with ADA-free CD26, which, either directly or via immunocapitation is immobilized onto a solid carrier, be 20 used for determining adenosine deaminase in biological fluids by means of an inhibition test.

### 4.2. Method

Transparent polystyrene microtiter plates (Nunc, 25 Denmark) are coated overnight at 4°C with anti-CD26 by incubation with a solution of 3 µg/ml anti-TA5.9 in carbonate buffer, pH 9.6. The remaining free binding sites are blocked the next morning with 3% BSA.

A reference preparation, which contains 50 mU/ml 30 CD26, was prepared in a human albumin matrix SOPP (Belgian Red Cross). A series of standards was also made with bovine adenosine deaminase EC3.5.4.4 (Sigma A-1155) which contained 0 U/ml, 1 U/ml, 10 U/ml and 100 U/ml ADA, respectively.

The inhibition EIA is carried out as follows. In a 35 first step, 100 µl of the ADA-standards (0,1, 10 and 100 U/ml) are placed in the wells of the microtiter plate. Incubation is carried out for one hour at 37°C. Thereafter the plate is washed 5 x with PBS-buffer, pH 7.2. In a second

step 100  $\mu$ l of a biotinylated TA 5.9 conjugate, which is diluted in PBS-buffer pH 7.2, is added with 0.1% Tween 20 and 2.5% BSA. After incubation for one hour at 37°C, the plates are washed 5 x with washing buffer. Thereafter 100  $\mu$ l of streptavidin-HRP is added to each well. The streptavidin-HRP is diluted in the same way as the biotinylated antibody. After incubating for 20 minutes at 37°C the plates are again washed 5 x with washing solution after which 100  $\mu$ l of a  $H_2O_2$  substrate solution and 100  $\mu$ l chromogenic reagent (TMB) is added to each well. After incubating for 20 minutes at 37°C the reaction is stopped by the addition of 50  $\mu$ l 2 N  $H_2SO_4$ . Finally the optical density of the solution is determined at 450 nm.

#### 4.3. Results

The results of a standardization example of an ADA-concentration assay by means of inhibition EIA are represented in figure 16.

#### EXAMPLE 3

##### In vitro co-stimulating properties of the anti-CD26 monoclonal antibody anti TA5.9

##### 1. Introduction

Various plasma membrane proteins are related to T-cell activation. It is known that apart from the classical T-cell receptor-CD3-complex, depending on the experimental conditions, the triggering of CD2, CD5 and CD28 can contribute to T-cell activation. It is suspected that a co-mitogenic effect for the stimulation of CD4+ T-cells also exists for anti-CD26 monoclonal antibodies. In this example this effect of the anti-TA5.9 monoclonal antibody according to the invention on CD26-CD3 mediated activity of purified CD4+ and CD8+ T-cells is investigated. This way it is possible to compare the in vitro effects of anti-TA5.9 on CD4+ T-cells with other anti-CD26 monoclonal antibodies and to investigate the effect of CD26 triggering on CD8+ T-cells.

## 2. Method

The activation and proliferation of CD4+ and CD8+ T-cells were monitored via determining DNA-synthesis on the one hand and the expression of the activation markers on the cell surface on the other hand.

Human CD4+ and CD8+ T-cells were purified from peripheral blood (EDTA as anti-coagulant) by isolating PBMC via Ficoll-Paque density gradients, removing monocytes by adhering them to polystyrene petri-dishes (for 2 hours at 37°C), the positive selection of CD8+ T-cells by means of an immuno-magnetic carrier and the positive selection of CD4+ T-cells from the remaining cell population, also via an immuno-magnetic carrier. The purified cell populations from each donor were stimulated parallel and under identical conditions in the presence of the following immobilized stimuli:

- (1) Control monoclonal antibody (anti-MHC class I monoclonal antibody)
- (2) anti-CD3 (OKT3) monoclonal antibody and control monoclonal antibody
- (3) anti-CD26 monoclonal antibody (TA5.9 or TA1)
- (4) anti-CD3 and anti-CD26 monoclonal antibody (TA5.9 or TA1)

The expression of the activation marker CD69, CD25, CD71 and HLA-DR on CD4+ and CD8+ T-cells was determined at different moments during the activation. Their CD45RO+ (memory) and CD45RO- (naive subsets) were also determined flow cytometrically (FACScan). The cell proliferation was measured by means of <sup>3</sup>H-thymidine incorporation (in quadruplicate) after 5 days. The cell populations obtained have an average purity of 96% for the CD4+ T-cells and 95% for the CD8+ T-cells.

## 3. Results

The anti-CD26 monoclonal antibodies Ta1 and TA5.9 cannot produce any proliferation on their own. The immobilized anti-CD3 alone in some individuals caused a significant proliferation of CD8+ T-cells but never of CD4+

T-cells. A co-stimulation via CD26 (TA5.9 or Ta1) involves a strong proliferation of CD4+ and CD8+ T-cells. Cyclosporin blocks the T-cell proliferation which is caused by the CD3-CD26-co-stimulation.

5           The expression of the activation markers on CD4+ and CD8+ T-cells is as follows:

          Anti-CD3 induced a significant expression of CD69 on purified CD8+ T-cells, whilst for the CD4+ T-cells, only a small and late increase is to be seen. In a similar  
10 manner, but less clearly, the activation markers CD25, and in some individuals also CD71, are expressed on CD8+ T-cells but not on CD4+ T-cells after anti-CD3 stimulation. An important co-stimulating effect of anti-CD26 monoclonal antibodies (Ta1 and TA5.9) is observed on the CD3 mediated  
15 activity. The expression of CD69 on CD4+ and CD8+ T-cells is within 24 hours after the start of this co-stimulation at the most. The expression of CD25 and CD71 also increases to more than 90% positive cells after 72 hours and this is also the case for the CD4+ and the CD8+ T-cells.

20           Considering the expression of activation markers on CD45RO+ and RO- subsets of CD4+ and CD8+ T-cells, the following has been found. After stimulation of CD8+ T-cells with anti-CD3, CD69 is expressed on both the CD45RO+ and CD45RO- subsets. This expression is however twice as high on  
25 the CD45RO+ CD8+ T-cells in comparison to the CD45RO- CD8+ T-cells. CD25 is mainly expressed on CD45RO+ CD8+ T-cells. Co-stimulation via CD26 results in a comparable increase of CD69 and CD25 expression both on memory and naive CD4+ and CD8+ T-cells. The up-regulation of CD25 occurs much quicker  
30 on the CD45RO+ cells.

          Another much used parameter of T-cell activity is the expression of HLA-DR. Under control conditions the HLA-DR within the CD4+ T-cells is almost exclusively present on the CD45RO+ subsets. Within the CD8+ cell population HLA-DR  
35 is mainly, but not exclusively, present on the CD45RO+ cells. The up-regulation of HLA-DR expression on the CD4 and CD8+ T-cells after CD3-CD26 co-stimulation, occurs quicker

and is more apparent on the CD45RO+ subset of both T-cell populations.

#### 4. Conclusion

5           It can generally be assumed that a strong co-stimulating effect is observed of anti-CD3 and anti-CD26 monoclonal antibodies on purified CD4+ and CD8+ T-cells. The expression of activation markers is more accelerated on CD8+ in comparison with CD4+ T-cells and on CD45RO+ with respect  
10 to CD45RO- subsets. The co-stimulatory effect of anti-TA5.9 is in general clearly higher than that of anti-Ta1.

#### 5. Description of the figures

          In figure 9a the co-stimulation of purified CD8+  
15 and CD4+ T-cells by 1 µg/ml immobilized anti-CD3 (OKT3) in combination with a control monoclonal (open blocks) or anti-TA5.9 (hatched blocks) respectively, is shown. Figure 9b gives result for 10 µg/ml OKT3.

          Figures 10 and 11 give again the co-stimulation of  
20 purified CD4+ and CD8+ T-cells by 1 µg/ml (a) and 10 µg/ml (b) immobilized anti-CD3 (OKT3) respectively in combination with a control monoclonal (open blocks), anti-TA5.9 (hatched blocks), or anti-Ta1 (stippled blocks) respectively.

          Figures 12-15 show the expression of activation  
25 markers after CD3 stimulation and CD3-CD26 co-stimulation of purified CD4+ and CD8+ T-cells. At the given times, the expression of the activation-antigen (x-axis, FITC-fluorescence) is determined on the CD45RO+ and CD45RO- cells (y-axis, PE-fluorescence) with the aid of direct  
30 immunofluorescence.

          The table gives a summary of the expression of activation markers on the different T-cell subsets after CD3-CD26 co-stimulation.



TABLE 1

Expression of activation markers on purified human CD4+ T-cells (T4-cells) and CD8+ T-cells (T8-cells), after co-stimulation with anti-CD3 (OKT3) and anti-CD26 (anti-TA5.9) monoclones.

	i.p. CD69 %		i.p. CD25 %			i.p. CD71 %		
	3 h	18 h	18 h	48 h	72 h	18 h	48 h	72 h
T4	52 (26)	83 (28)	55 (2,4)	87 (3,8)	97 (5,1)	24 (3,6)	76 (38)	93 (72)
CD45RO+	62 (21)	81 (27)	69 (2,1)	89 (3,0)	96 (4,0)	23 (23)	79 (26)	96 (48)
CD45RO-	43 (43)	84 (42)	37 (4,1)	81 (5,8)	97 (6,9)	25 (>100)	66 (82)	94 (>100)
T8	45 (3,5)	83 (2,6)	37 (4,4)	77 (3,0)	80 (2,5)	32 (4,6)	66 (3,7)	77 (3,0)
CD45RO+	40 (2,2)	87 (1,7)	58 (2,8)	90 (1,8)	93 (1,8)	31 (1,9)	76 (2,3)	92 (2,0)
CD45RO-	48 (4,8)	81 (4,0)	25 (11)	65 (5)	76 (3,5)	31 (15)	48 (9,6)	76 (7,6)

i.p.: the values are given as the cell percentage that the respective activation marker on the specified lymphocyte-subset (i.e. % on CD4+ subset, % on CD4+CD45RO+ and CD4+CD45RO- subsets)

(x): increase in positive cells in comparison with CD3 stimulation alone.

## REFERENCES

1. Gibley, E.R., Anderson, J.E., Cohen, F., Pollara, B. & Meuwissen, H.J. *Lancet* 1974. 2:1067.
- 5 2. Van Der Weyden, M. & Kelley, W.N. *J. Biol. Chem.* 1976. 251:5448.
3. Chechik, B.E., Schrader, W.P. & Minowada, J. *J. Immunol.* 1981. 126:1003.
4. Swallow, D.M., Evans, L. & Hopkinson, D.A. *Nature* 1977. 269:261.
- 10 5. Hirschhorn, R. *J. Clin. Invest.* 1975. 55:661.
6. Daddona, P.E. & Kelley, W.N. *Mol. Cell. Biochem.* 1980. 29:91.
7. Dinjens, W.N.M., ten Kate, J., van der Linden, E.P.M., Wijnen, J.T., Khan, P.M. & Bosman, F.T. *J. Histochem. Cytochem.* 1989.37:1869.
- 15 8. Morrison, M.E., Vijayasaradhi, S., Engelstein, D., Albino, A.P. & Houghton, A.N. *J. Exp. Med.* 1993. 177:1135.
- 20 9. Vanhoof, G., De Meester, I., van Sande, M., Scharpé, S. & Yaron, A. *Eur. J. Clin. Chem. Clin. Biochem.* 1992. 30:333.
10. Ulmer, A., Mattern, T., Feller, A.C., Heymann, E., & Flad, H-D. *Scand. J. Immunol.* 1990 31:429.
- 25 11. Bednarczyk, J.L., Carroll, S.M., Marin, C. & McIntyre, B.W. *J. Cell. Biochem.* 1991. 46:206.
12. Dang, N.H., Torimoto, Y., Deusch, K., Schlossman, S.F. & Morimoto, C. *J. Immunol.* 1990. 144:4092.
13. Schön, E., Sigbert, J., Kiessig, S.T., Demuth, H.-U., Neubert, K., Barth, A., Von Baehr, R. & Ansorge, S. *Eur. J. Immunol.* 1987. 17:1821.
- 30 14. Flentke, G.R., Munoz, E., Huber, B.T., Plaut, A.G., Kettner, C.A. & Bachovchin, W.W. *Proc. Nat. Acad. Sci. USA* 1991. 88:1556.
- 35 15. Tanaka, T., Kameoka, J., Yaron, A., Schlossman, S.F. & Morimoto C. *Proc. Natl. Acad. Sci. USA.* 1991. 33:737

16. Mattern, T., Scholz, W., Feller, A.C., Flad, H.D. & Ulmer, A.J. *Scand. J. Immunol.* 1991. 33:737.
17. Hendriks, D., De Meester, I., Umiel, T., Vanhoof, G., van Sande, M., Scharpé, S. & Yaron, A. *Clin. Chim. Acta.* 1991. 196:87.
18. Boyum, A. Isolation of mononuclear cells & granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 1968. 21:77.
19. Scharpé, S., De Meester, I., Vanhoof, G., Hendriks, D., van Sande, M., Van Camp, K. & Yaron, A. *Clin. Chem.* 1988. 34:2299.
20. De Meester, I., Scharpé, S., Vanham, G., Bosmans, E., Heyligen, H., Vanhoof, G. & Corte, G. *Immunobiol.* 1993. 188:145.
21. Andy, R.J., Finstadt, C.L., Old, L.J., Lloyd, K.O. & Kornfeld, R. *J. Biol. Chem.* 1984. 259:12844.
22. Andy, R.J. & Kornfeld, R. *J. Biol. Chem.* 1984. 259:-9832.
23. Dinjens, W.N.M., van der Boon, J., ten Kate, J., Zeijlemaker, W.P., De Bruijn, C.H.M.M., Bosman, F.T. & Khan, P.M. *Adv. Exp. Med. Biol.* 1986. 95B:407.
24. Subramanyam, M., Gutheil, W.C., Bachovchin, W.W. & Huber, B.T. *J. Immunol.* 1993. 150:2544.
25. Van Ham et al.

- 26 -

## CLAIMS

1. Ligand or a functional derivative thereof, with  
at least one specificity for the adenosine deaminase (ADA)  
5 binding-site of CD26.

2. Ligand according to claim 1, characterized in  
that it is a monoclonal antibody.

3. Monoclonal antibody or functional derivative  
thereof, produced by the hybridoma cell line anti-CD26-  
10 (Ta5.9-CC1-4C8) having the deposit accession number ECACC  
93110419.

4. Ligand according to the claims 1-3,  
characterized in that the functional derivative is a  $F_{ab}$ -,  
 $F_{ab}$ -,  $F_{(ab)_2}$ -, a  $scF_v$  or  $F_v$ -fragment of the monoclonal  
15 antibody.

5. Ligand according to claims 1-3, characterized  
in that the functional derivative is a chimeric antibody.

6. Ligand according to claim 1, characterized in  
that it is a modified version of adenosine deaminase.

20 7. Ligand according to claims 1-6, characterized  
in that the functional derivative is a conjugate between the  
ligand or a functional component thereof and a secondary  
molecule.

8. Ligand according to claim 7, characterized in  
25 that the secondary molecule is selected from the group  
consisting of toxins, enzymes, medicines, radioactive  
markers, florescent markers, magnetic resonance markers,  
proteases, synthetic and natural polymers.

9. A monoclonal antibody according to claims 1-4,  
30 characterized in that the functional derivative is a  
bispecific antibody or functional component thereof.

10. Ligand according to the claims 1-9 for use as  
a therapeutic agent for stimulating proliferation of T-  
lymphocytes.

35 11. Ligand according to claims 1-9 for use as an  
immunosuppressing agent.

12. Ligand according to claims 1-9 for use as a  
diagnostic agent in immuno-enzymatic assays.

- 27 -

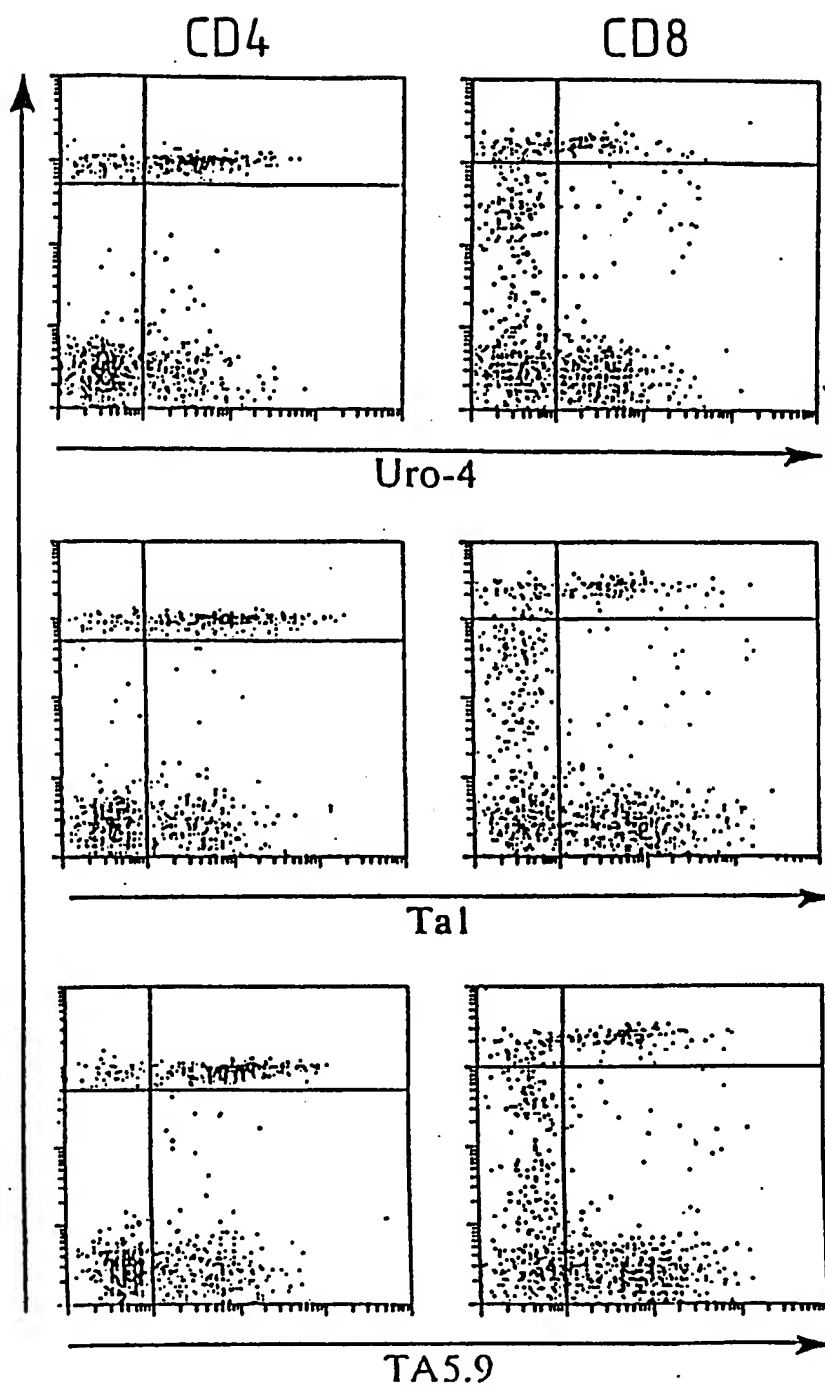
13. Ligand according to claims 1-9 for use as a diagnostic agent for monitoring malignantly transformed melanocytes.

14. Ligand according to the claims 1-9 for use as  
5 a diagnostic agent in imaging-techniques.

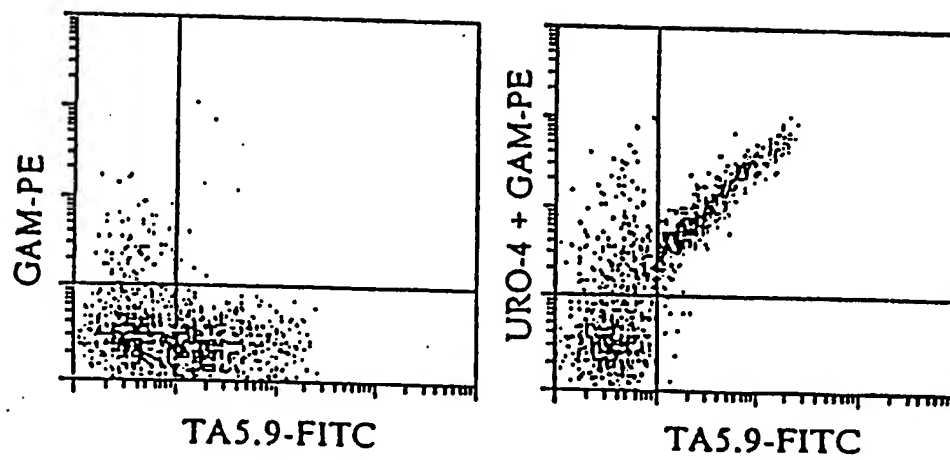
15. Ligand according to the claims 1-9 for use as a therapeutic agent against HIV-infection.

16. A Hybridoma cell line anti-CD26(TA5.9-CC1-4C8) having the deposit accession number ECACC 93110419.

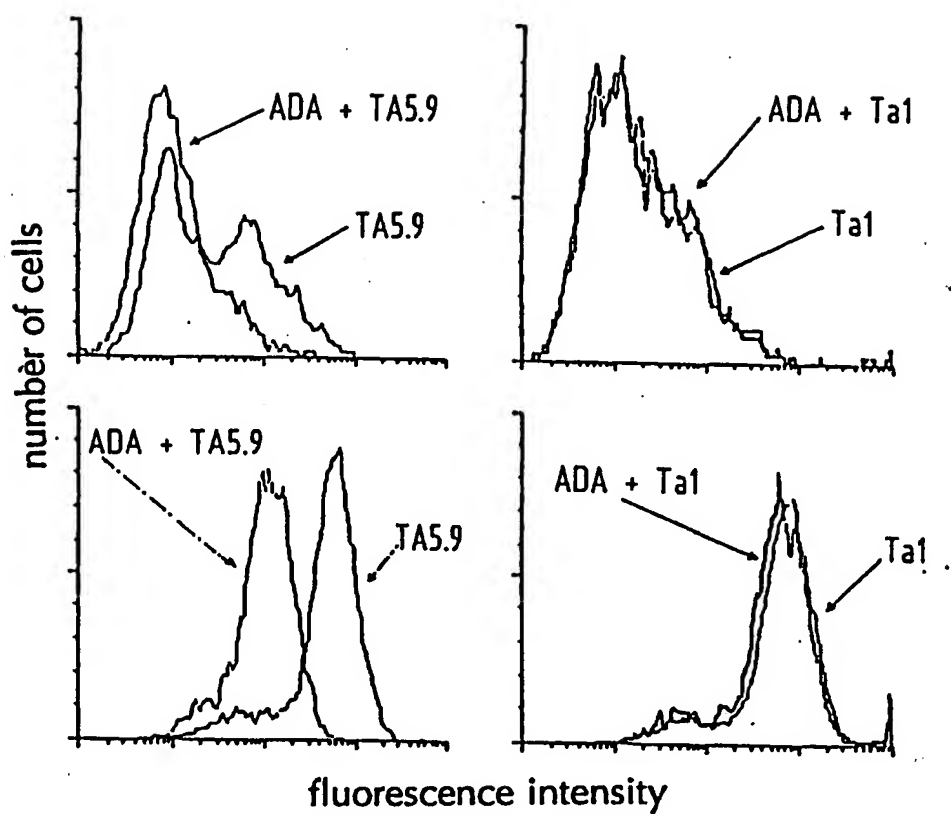
1/30

FIG. 1

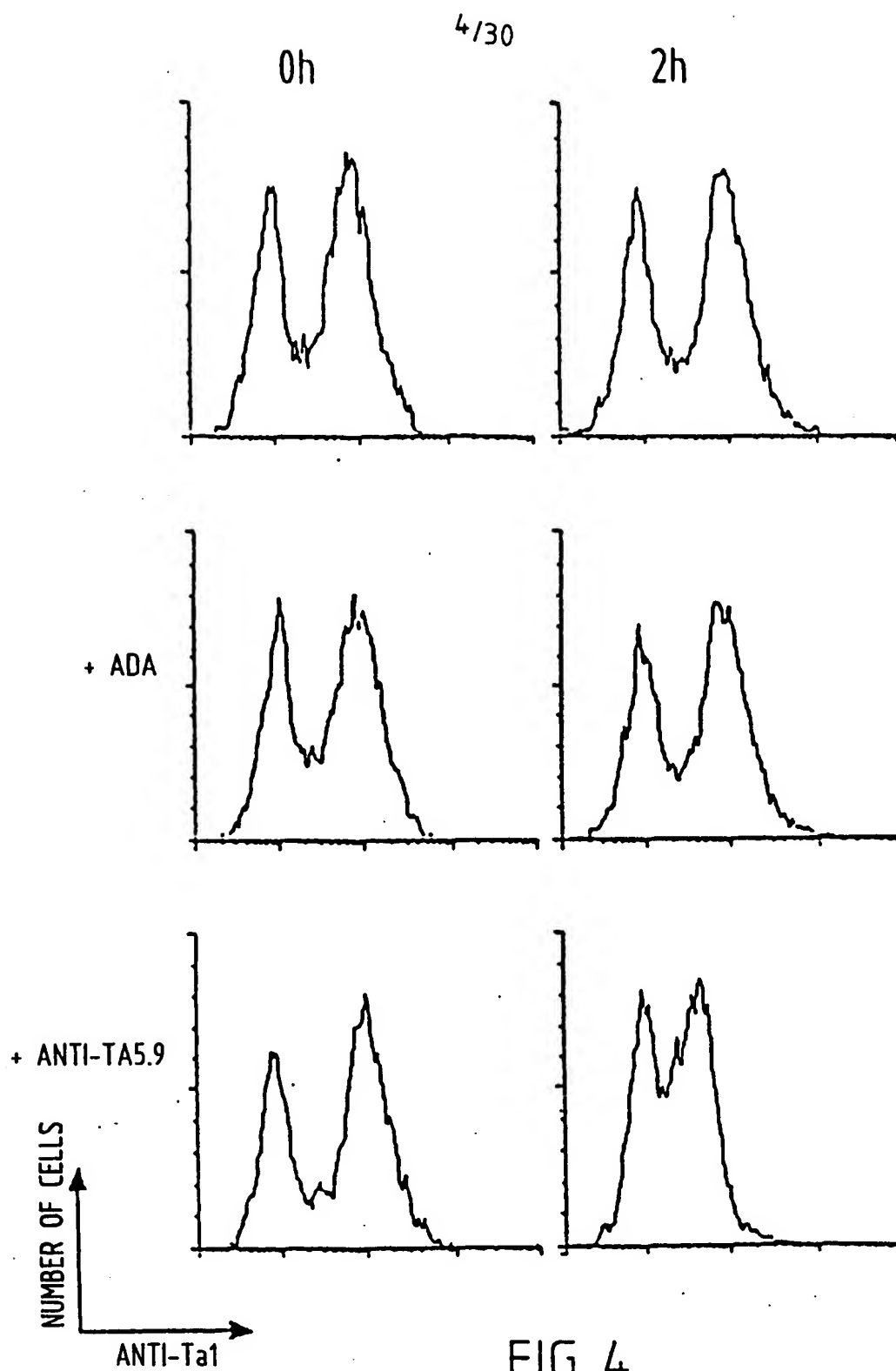
2/30

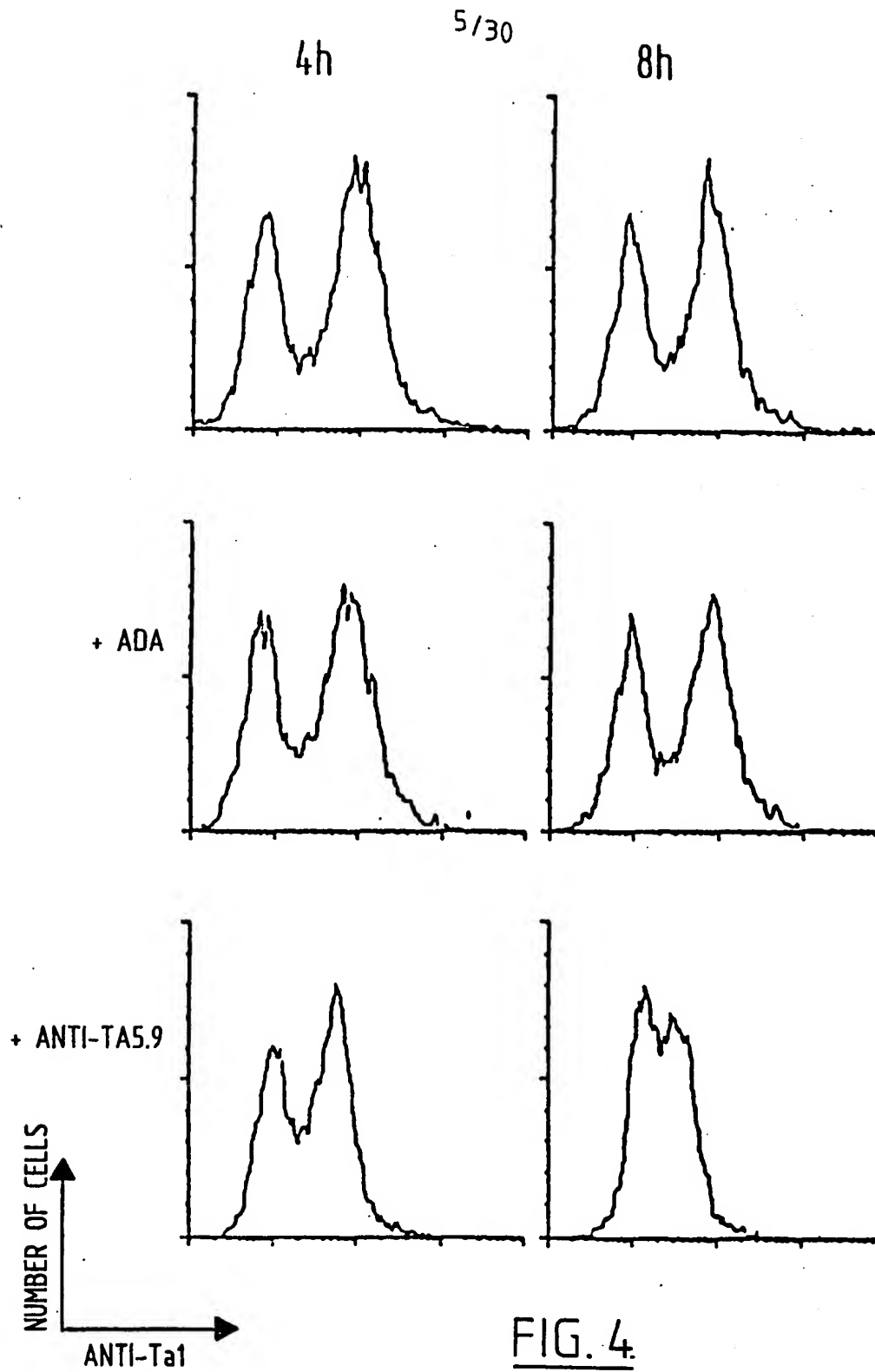
FIG. 2

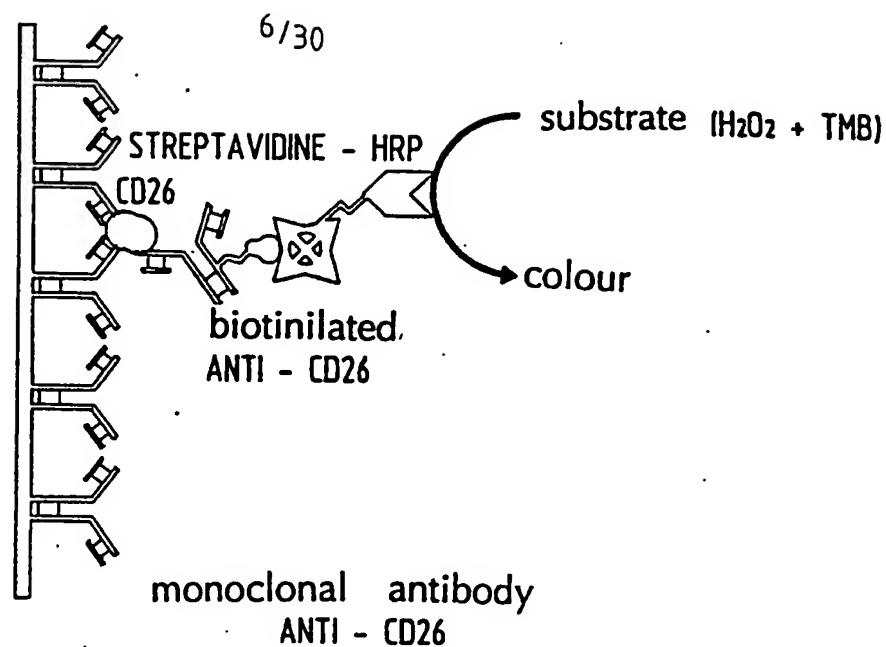
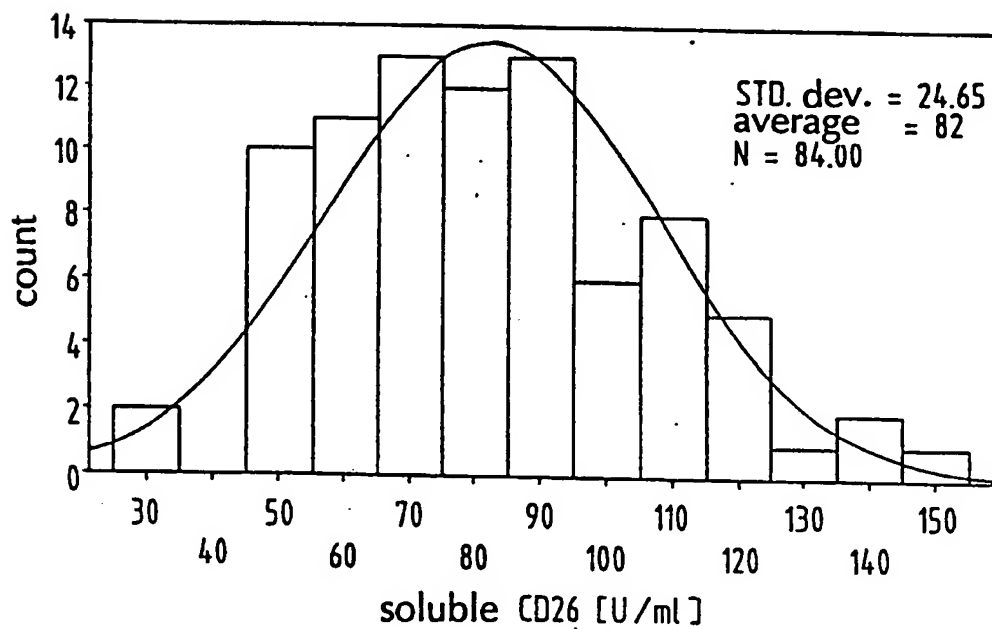
3/30

FIG. 3

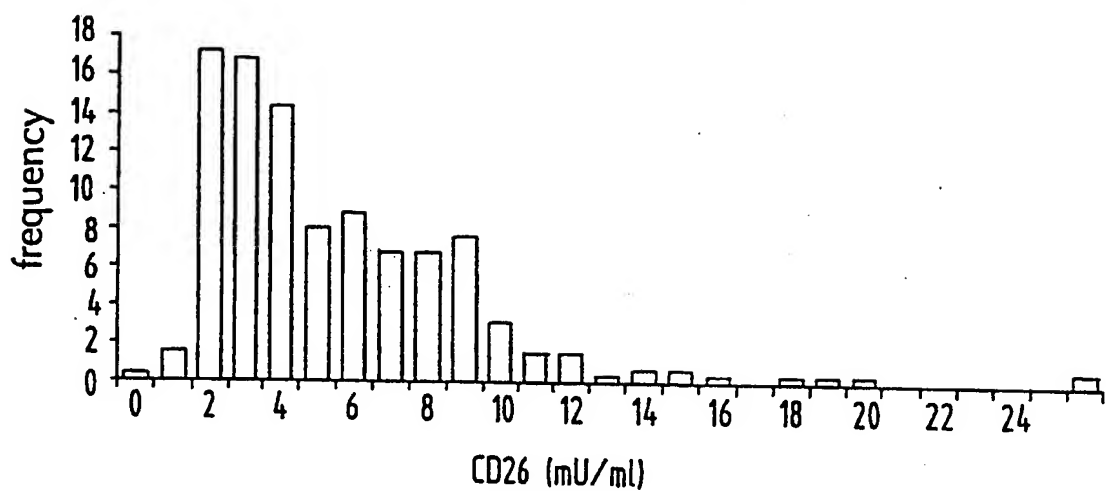
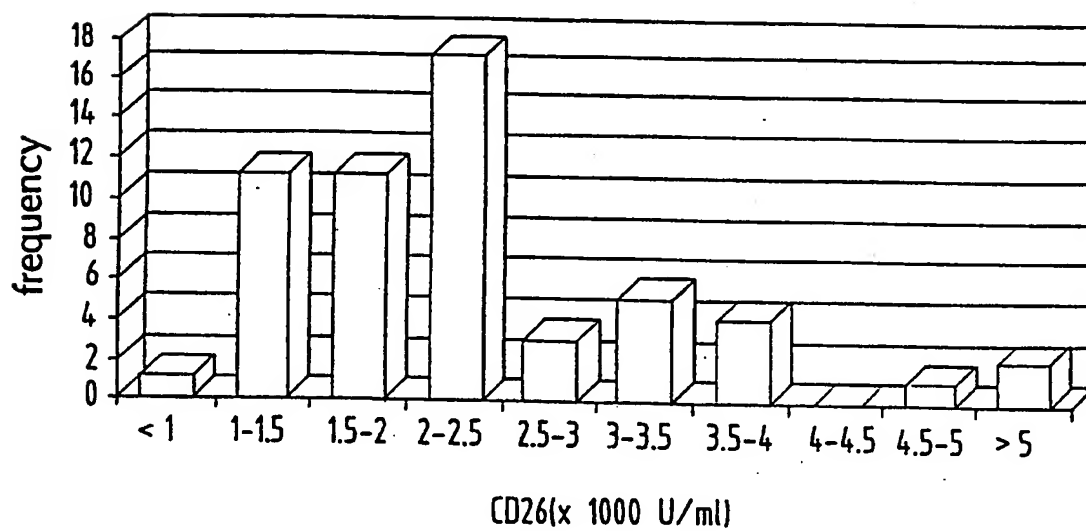


FIG. 4

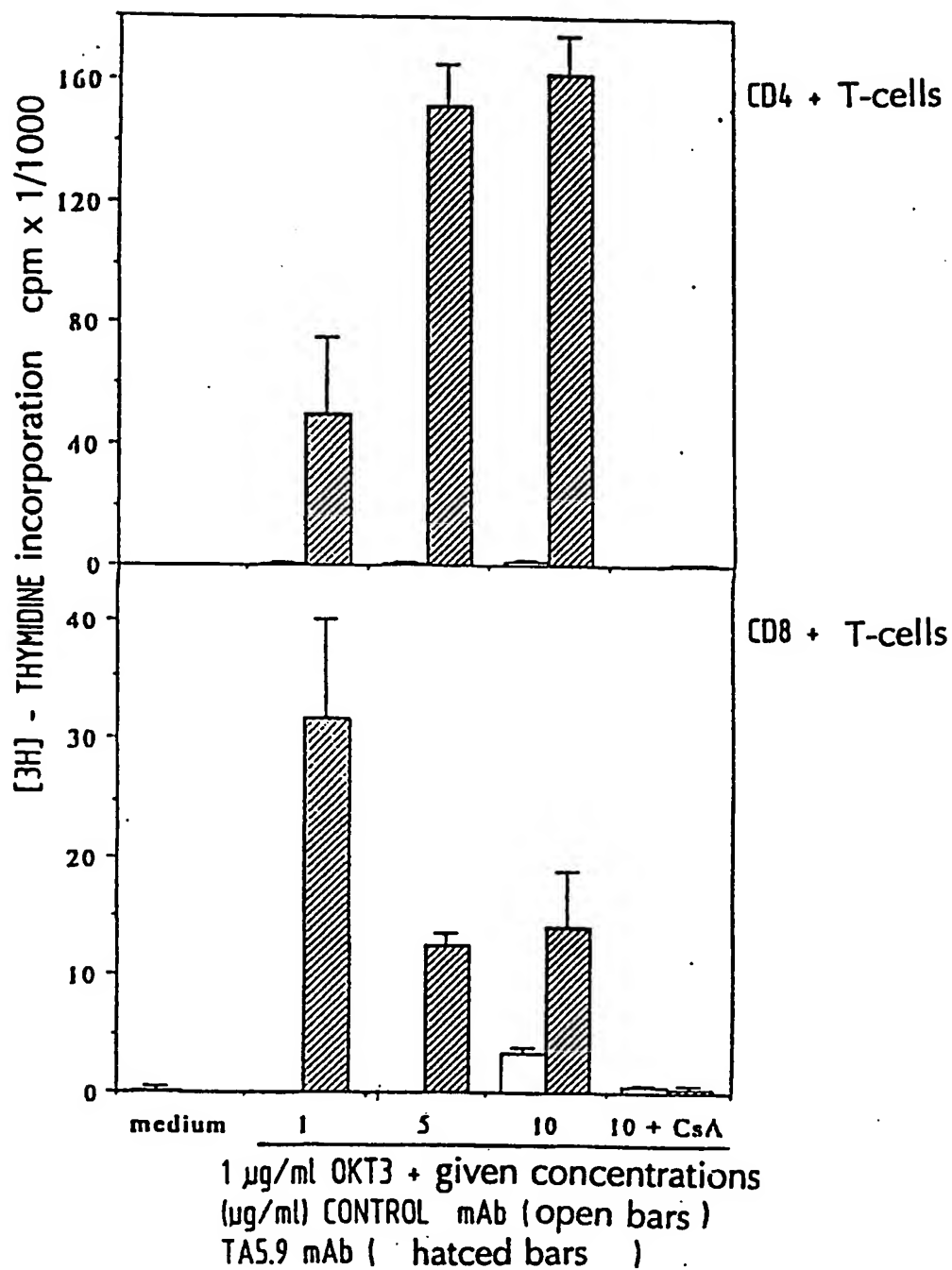


FIG. 5FIG. 6

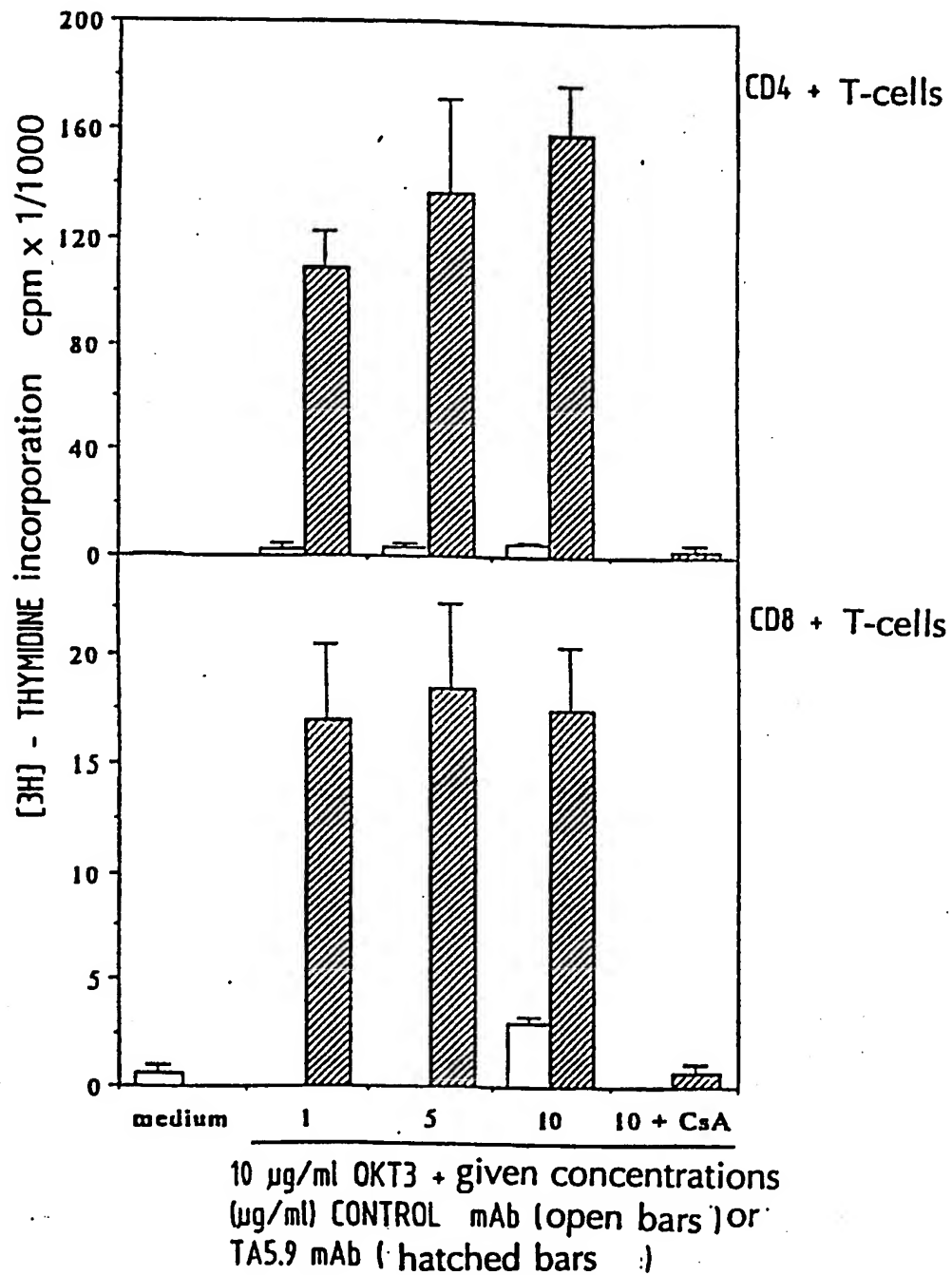
7/30

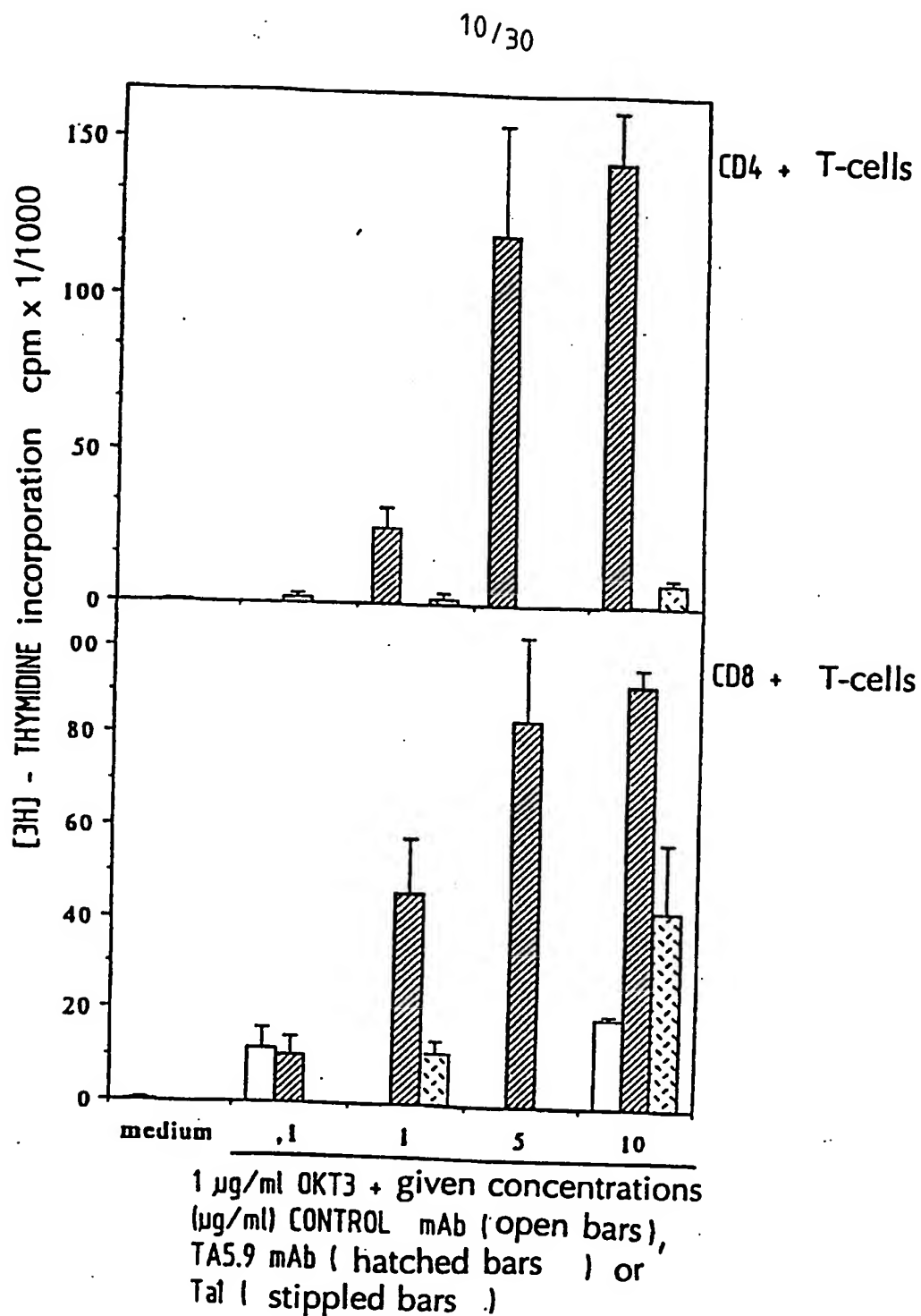
FIG. 7FIG. 8

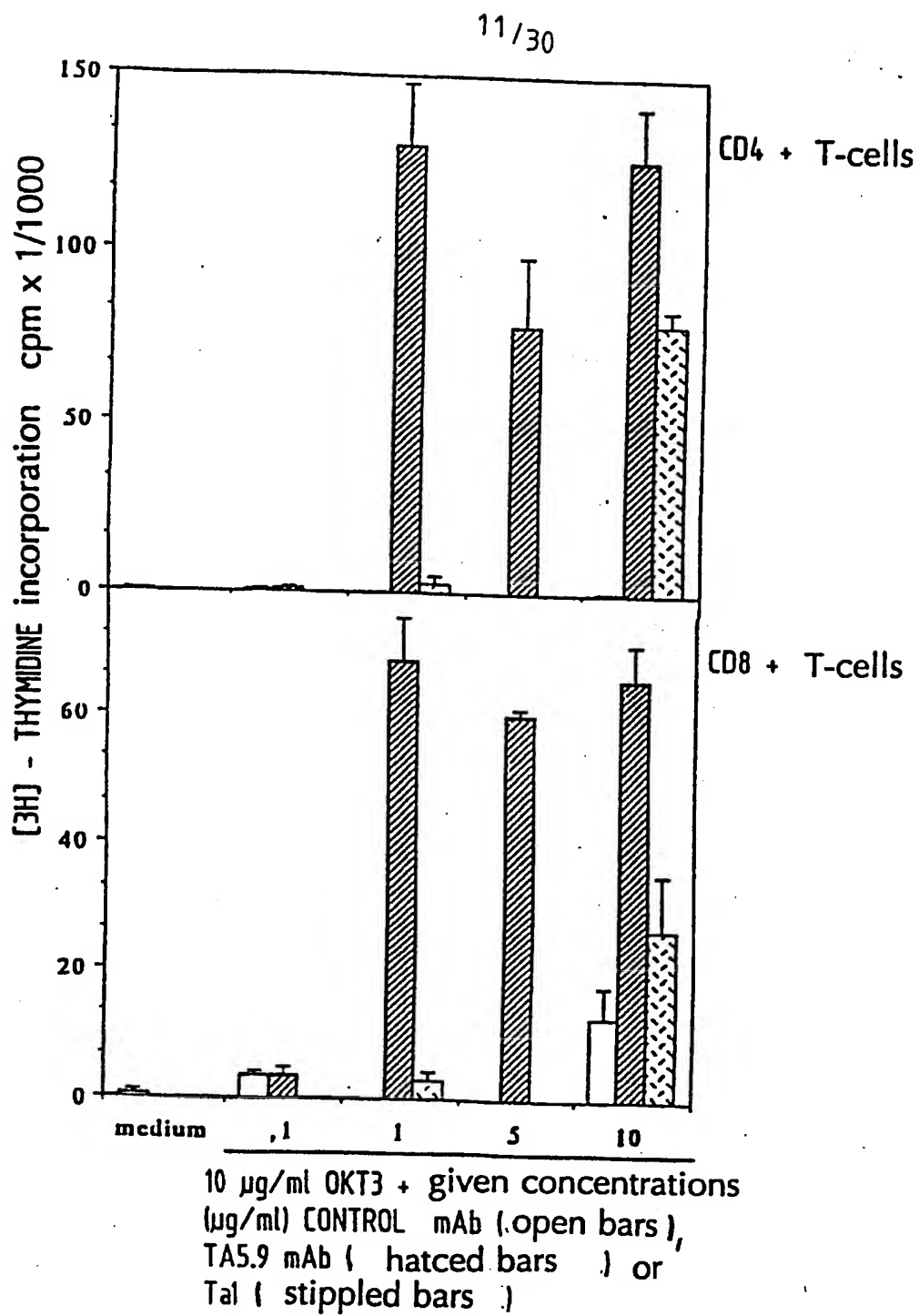
8/30

FIG. 9a

9/30

FIG. 9b

FIG. 10a

FIG. 10b



12/30

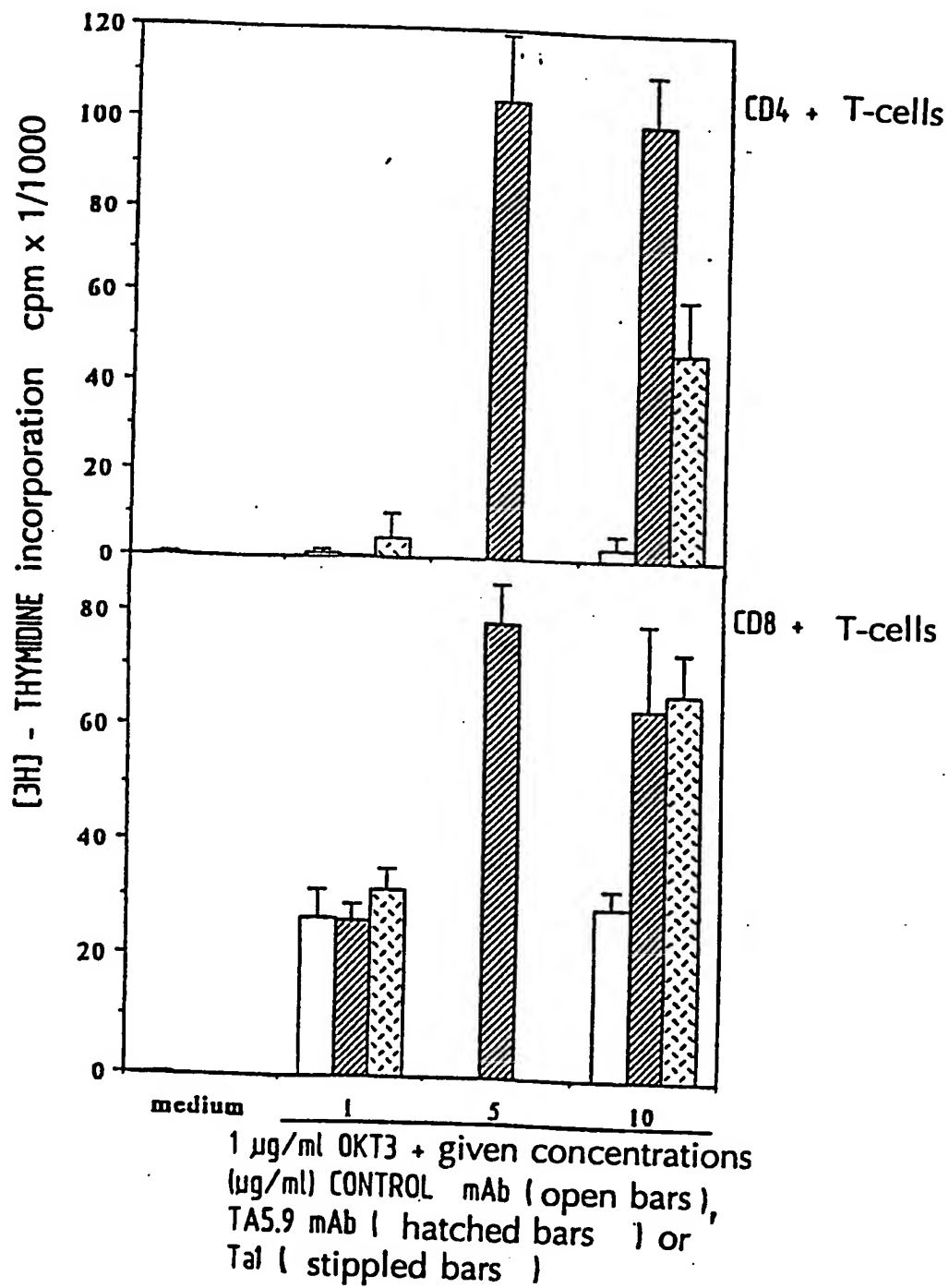
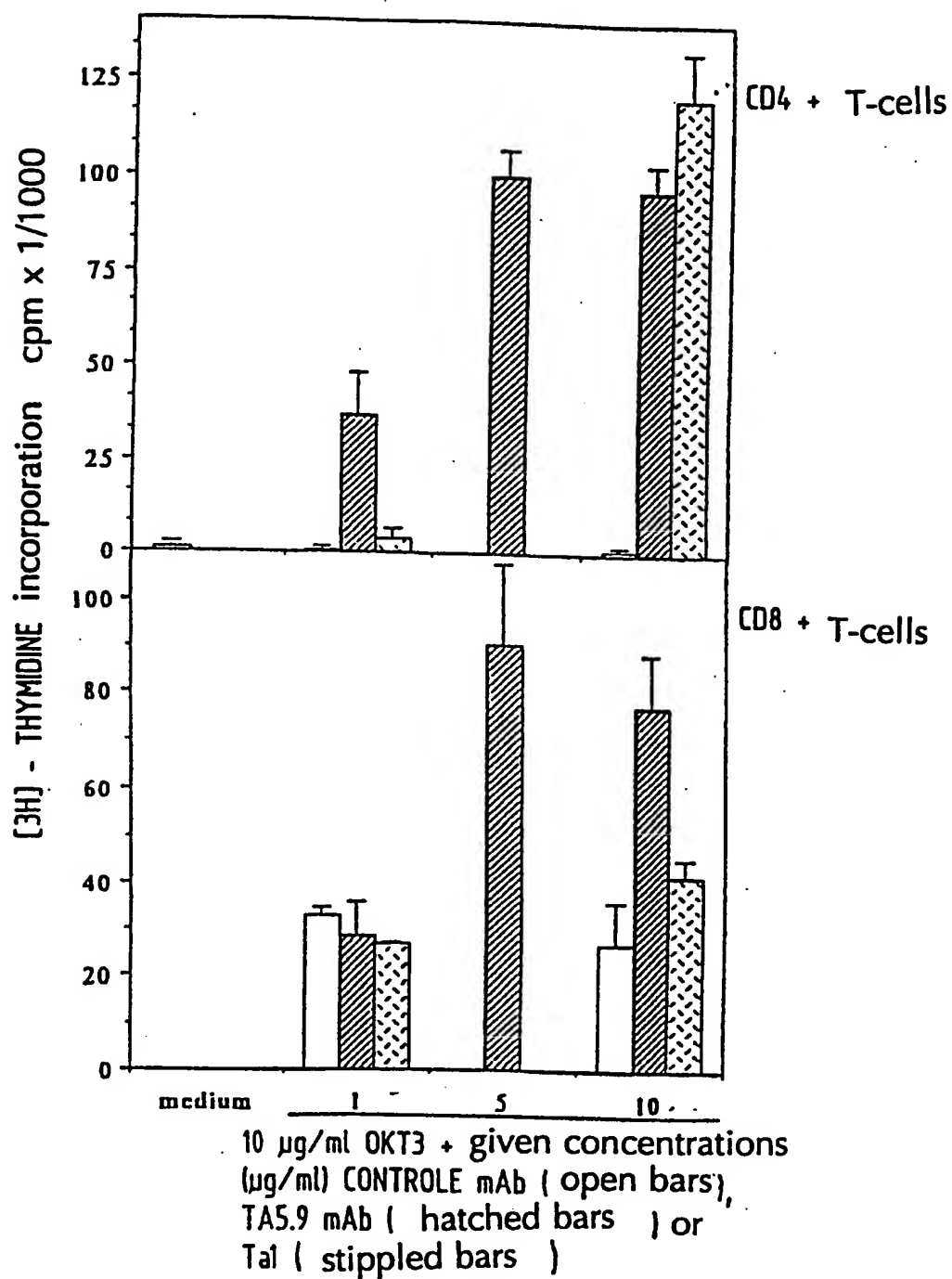


FIG. 11a

13/30

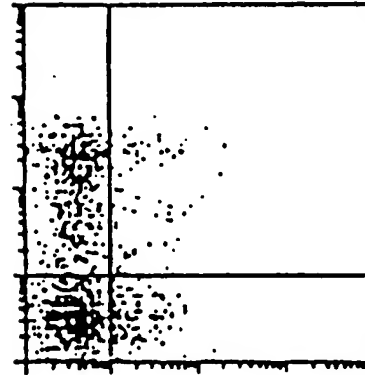
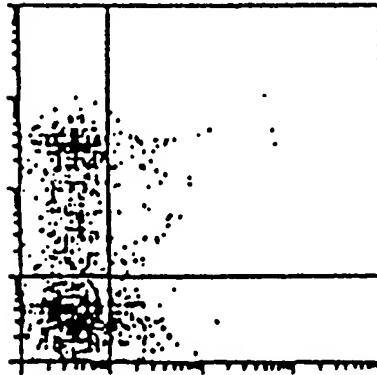
FIG. 11b

14/30

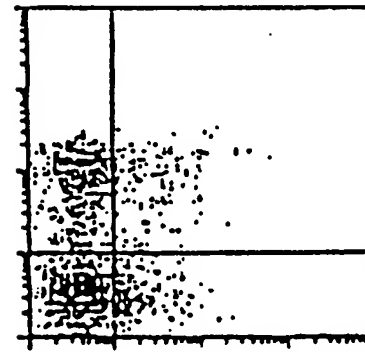
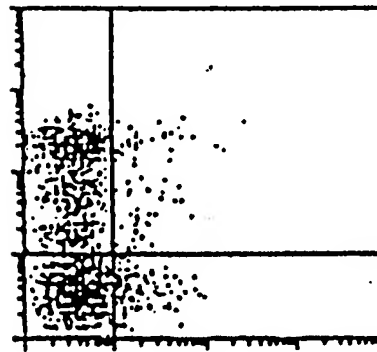
CONTROL

OKT3

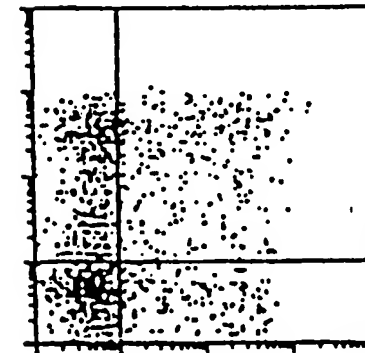
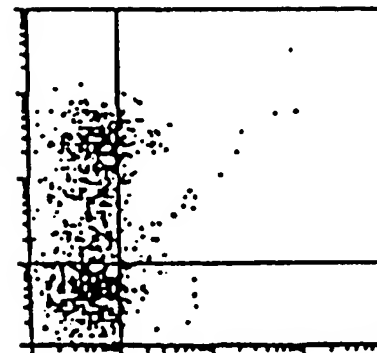
2h



4h



24h



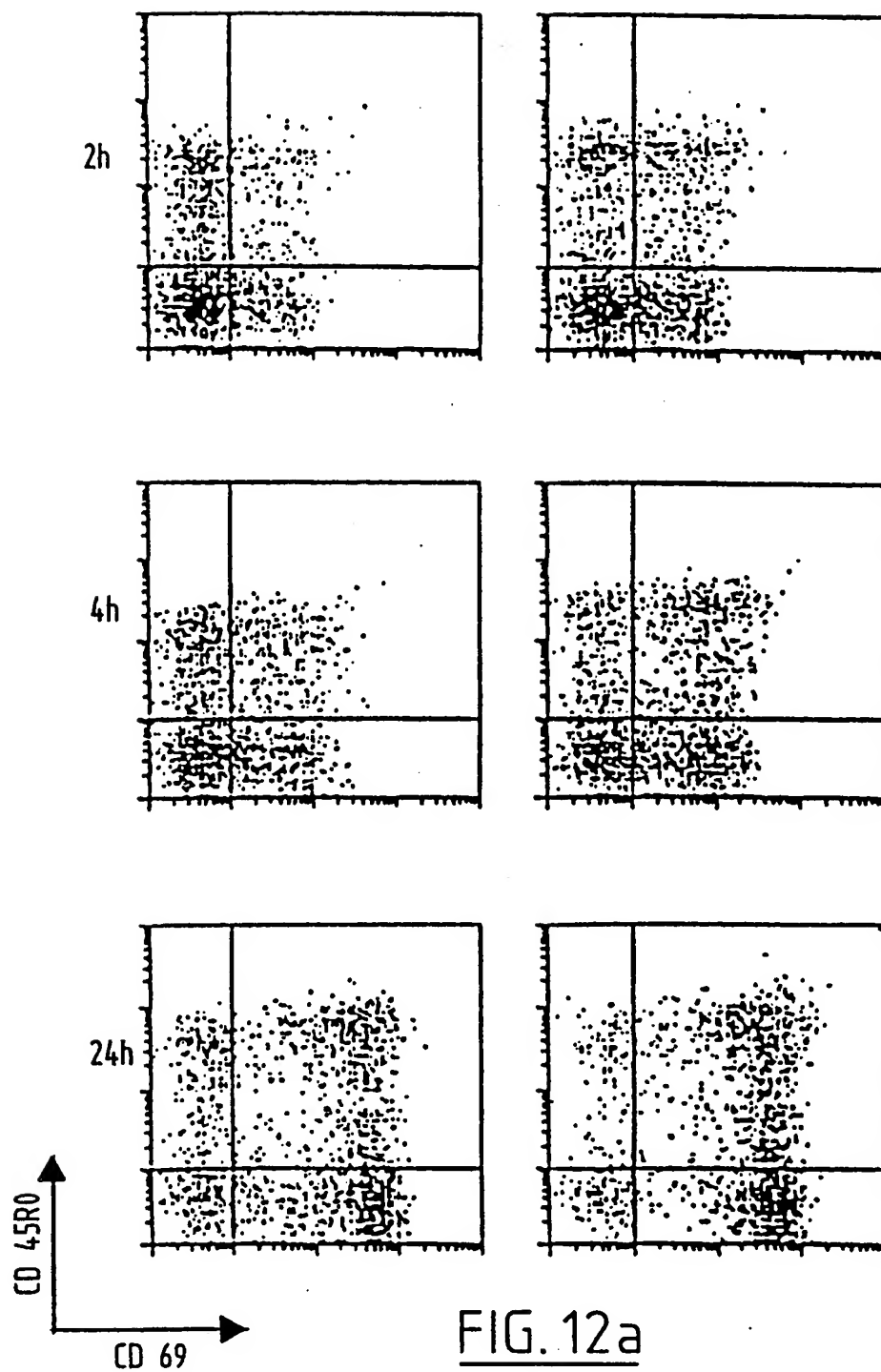
CD 45R0  
↑  
CD 69 →

FIG. 12a

15/30

OKT3/Ta1

OKT3/TA5.9



16/30

CONTROL

OKT3

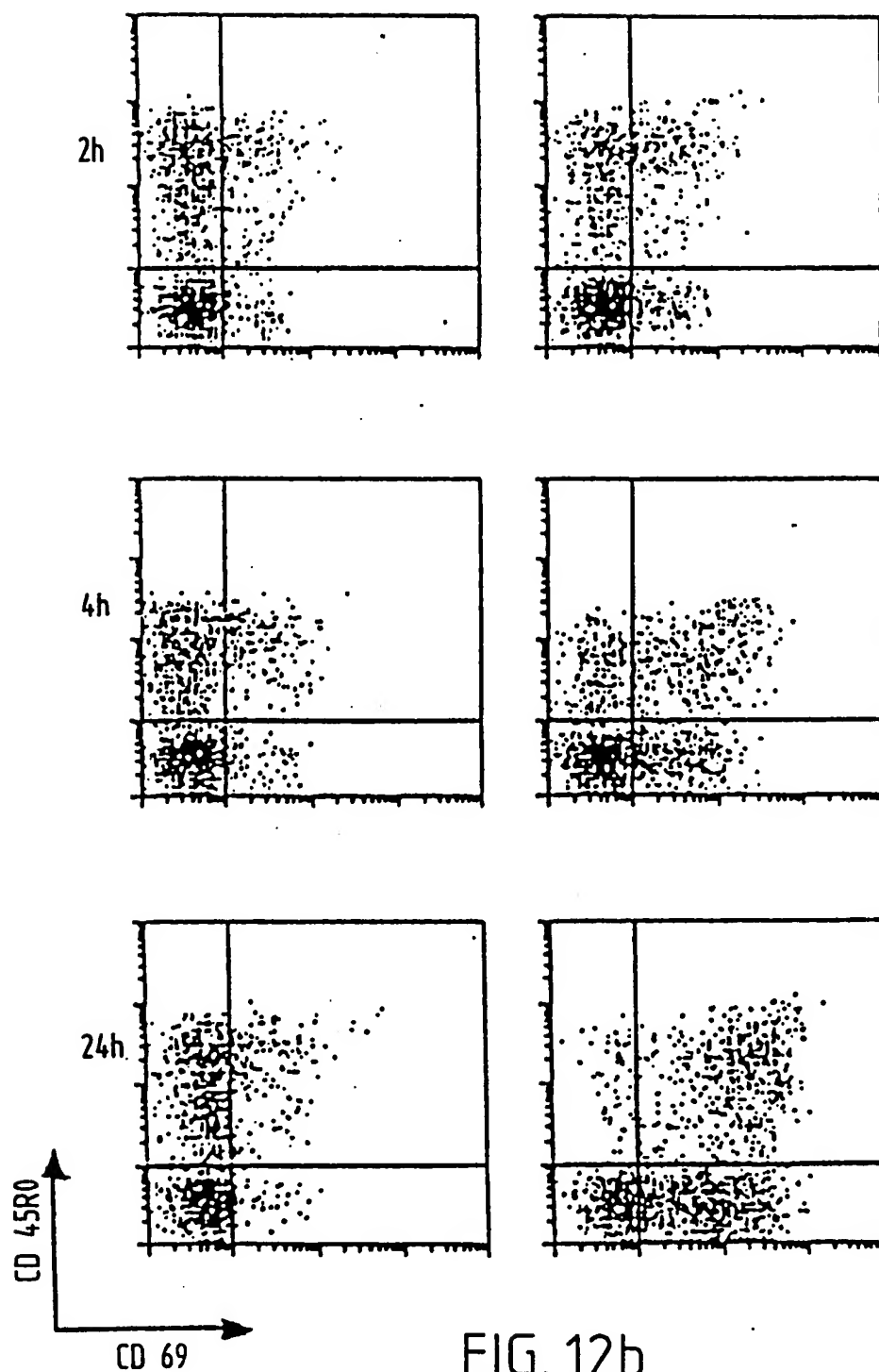
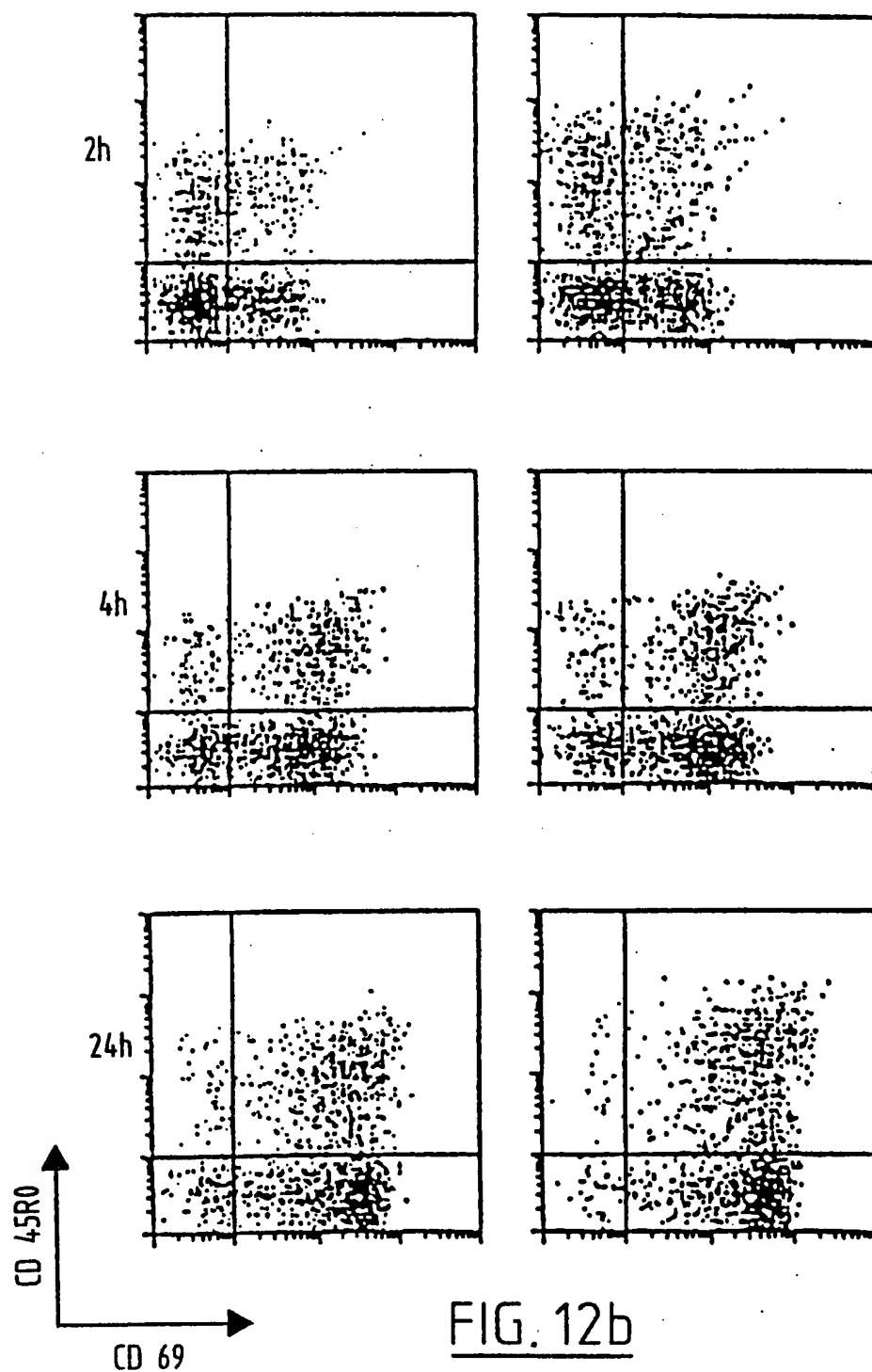


FIG. 12b

17/30

OKT3/Ta1

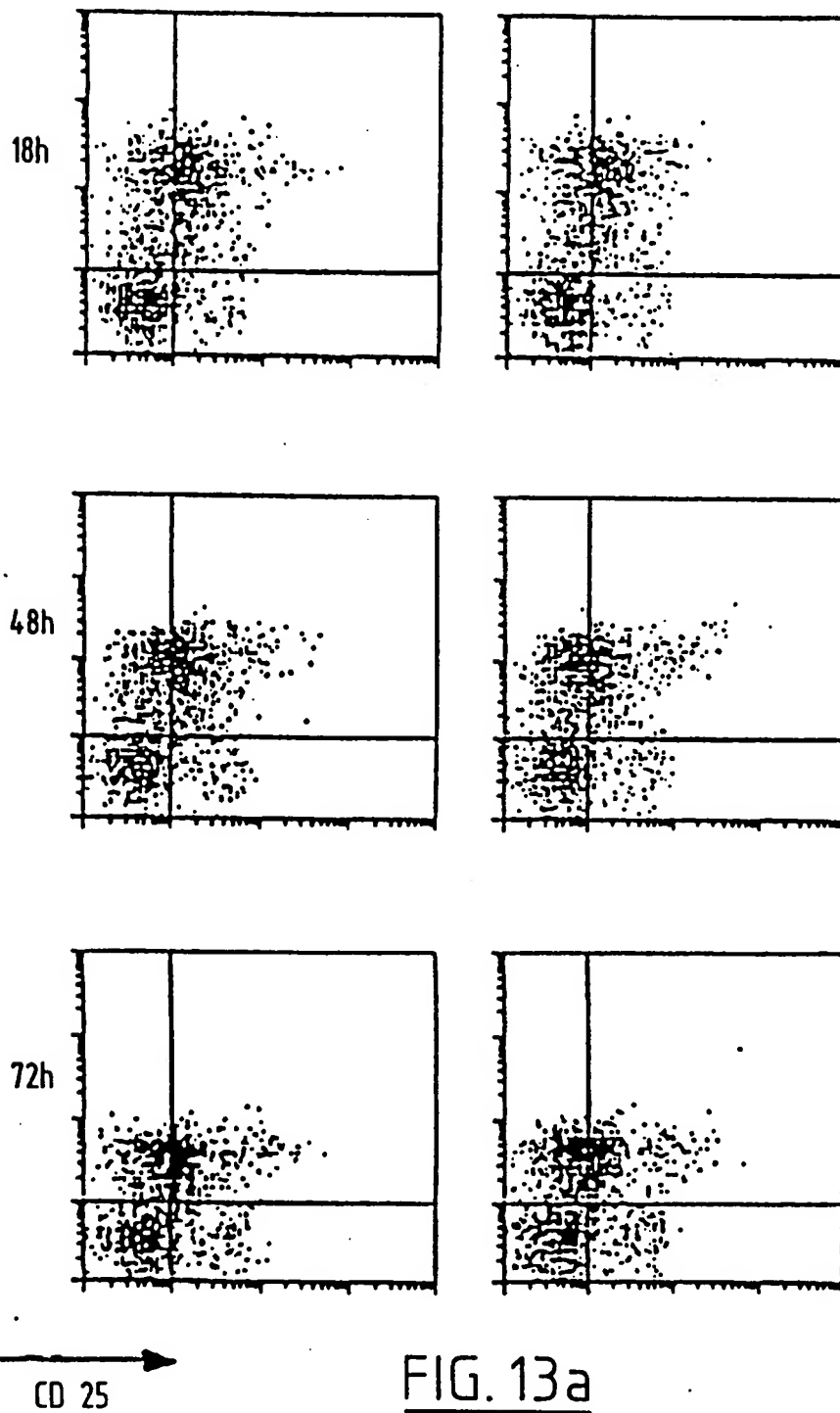
OKT3/TA5.9



18/30

CONTROL

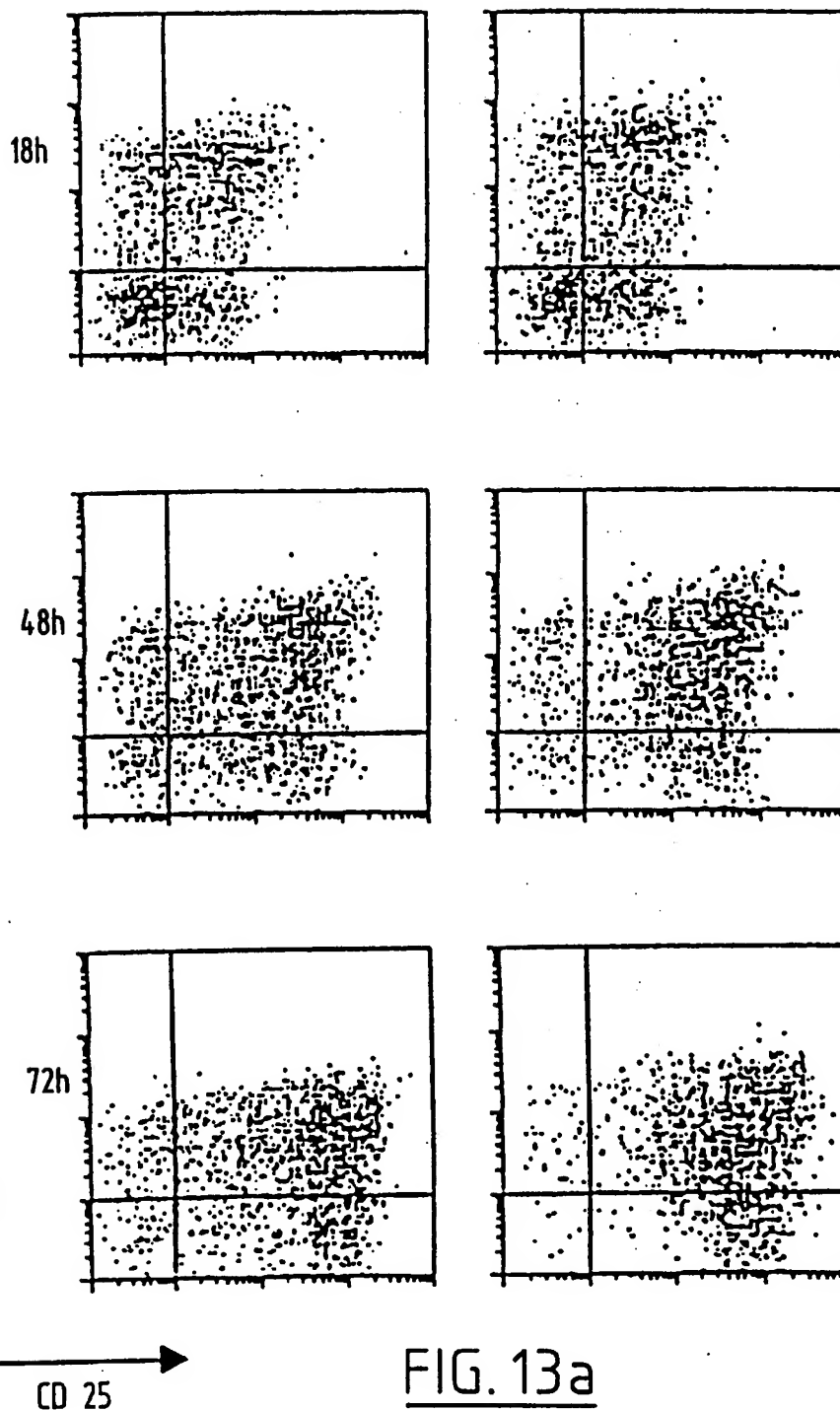
OKT3



19/30

OKT3/Ta1

OKT3/TA5.9

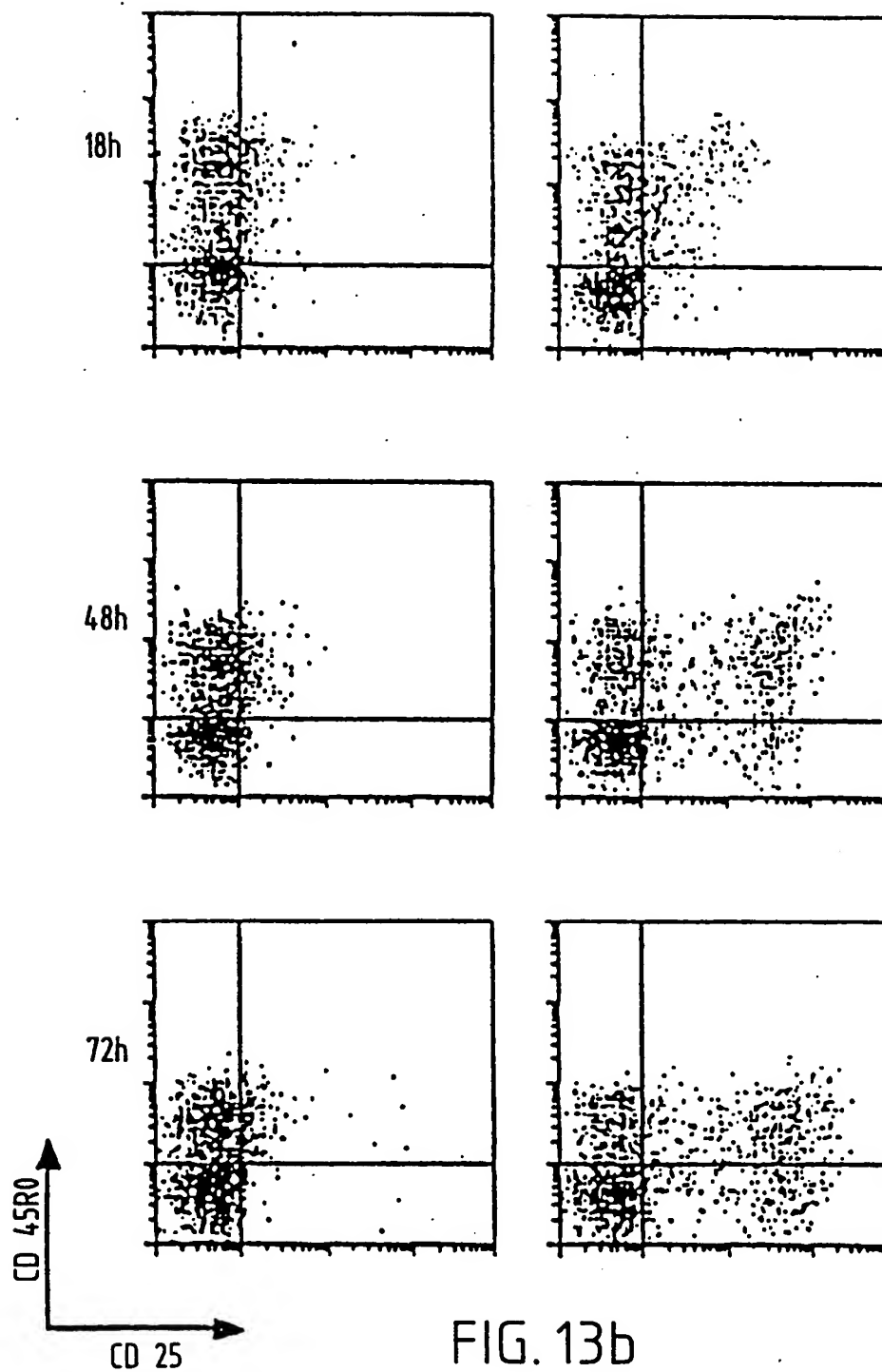




20/30

CONTROL

OKT3



21/30

OKT3/Ta1

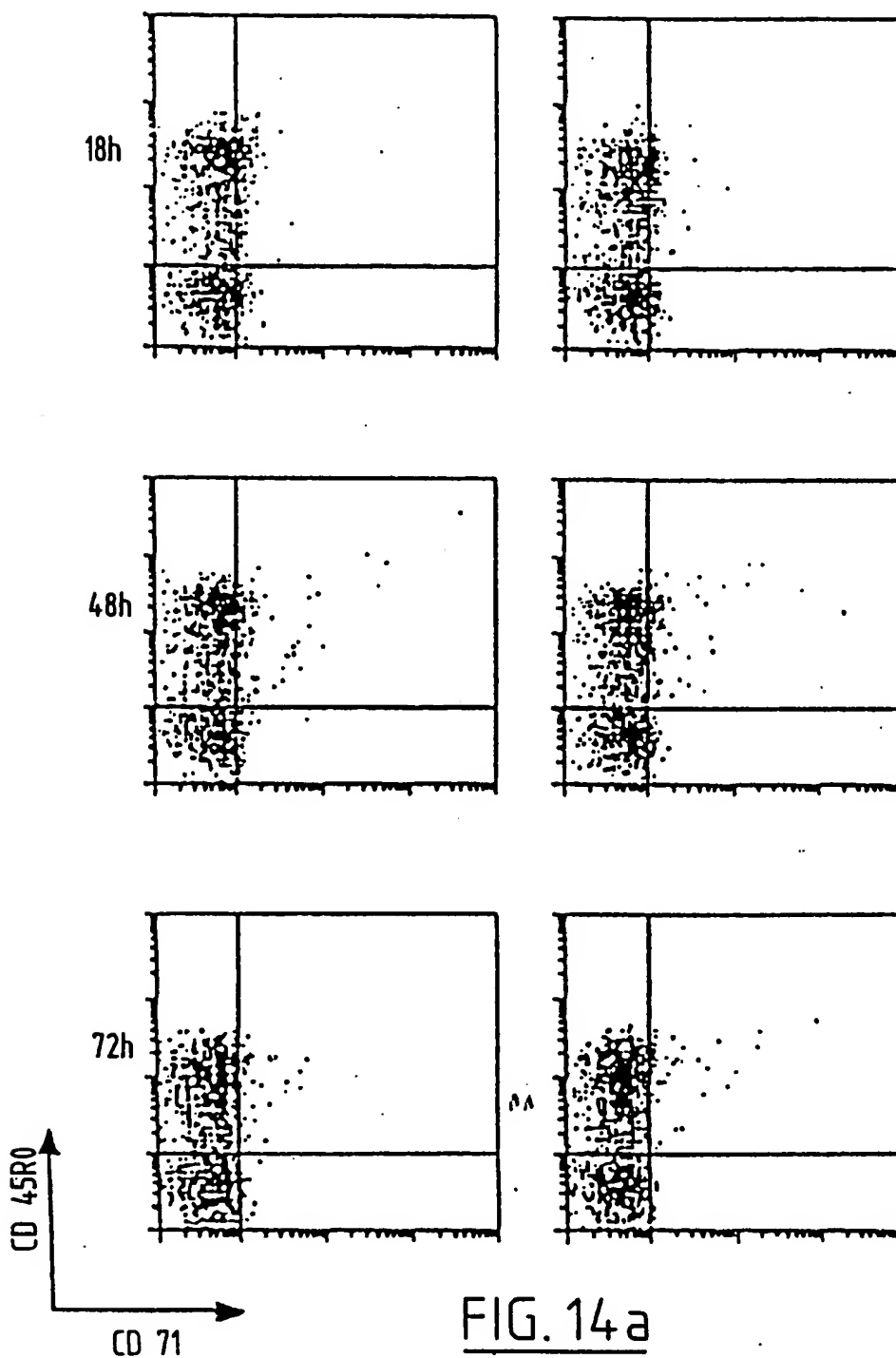
OKT3/TA5.9



22/30

CONTROL

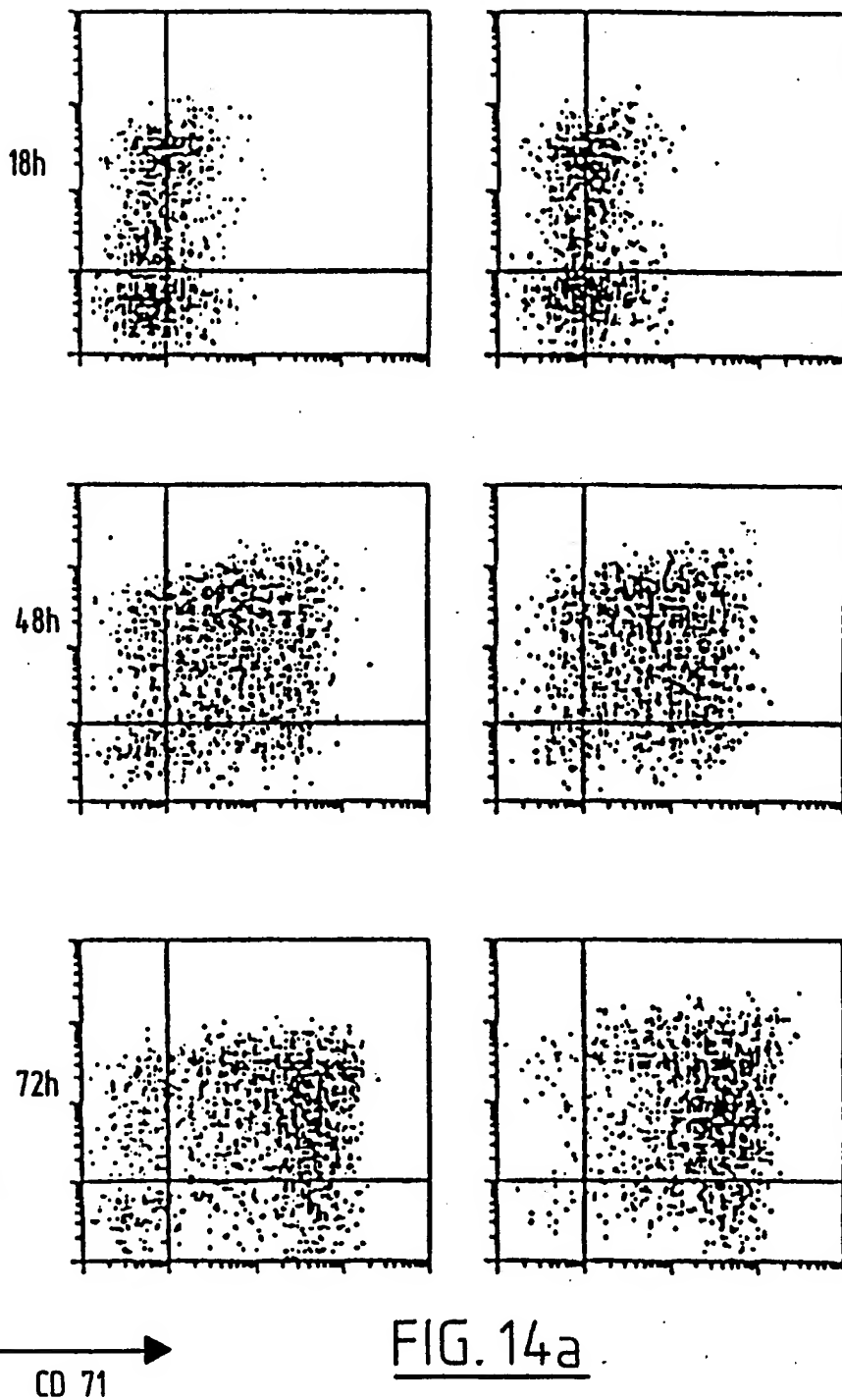
OKT3



23/30

OKT3/Ta1

OKT3/TA5.9

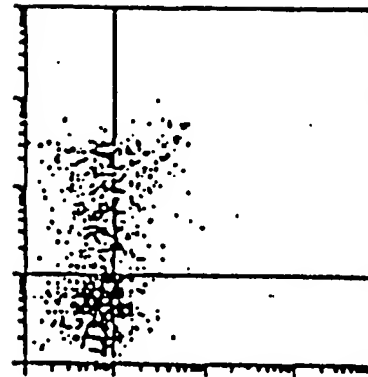
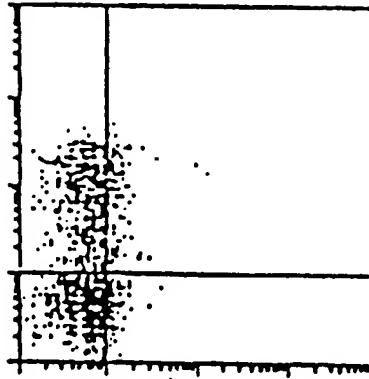


24/30

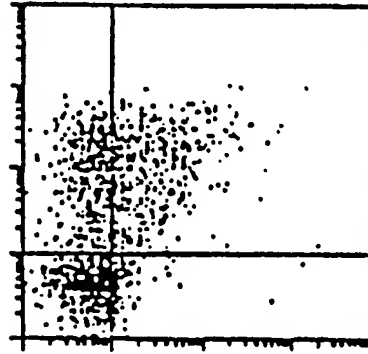
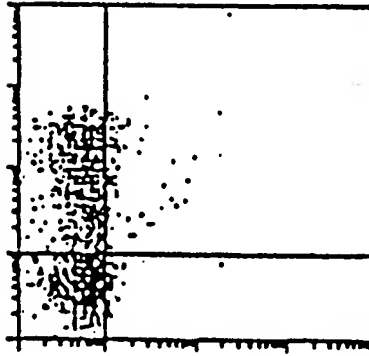
CONTROL

OKT3

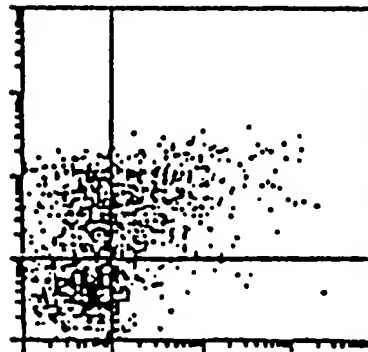
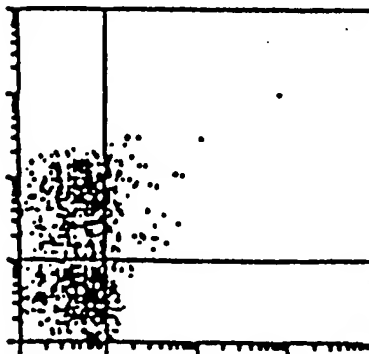
18h



48h



72h



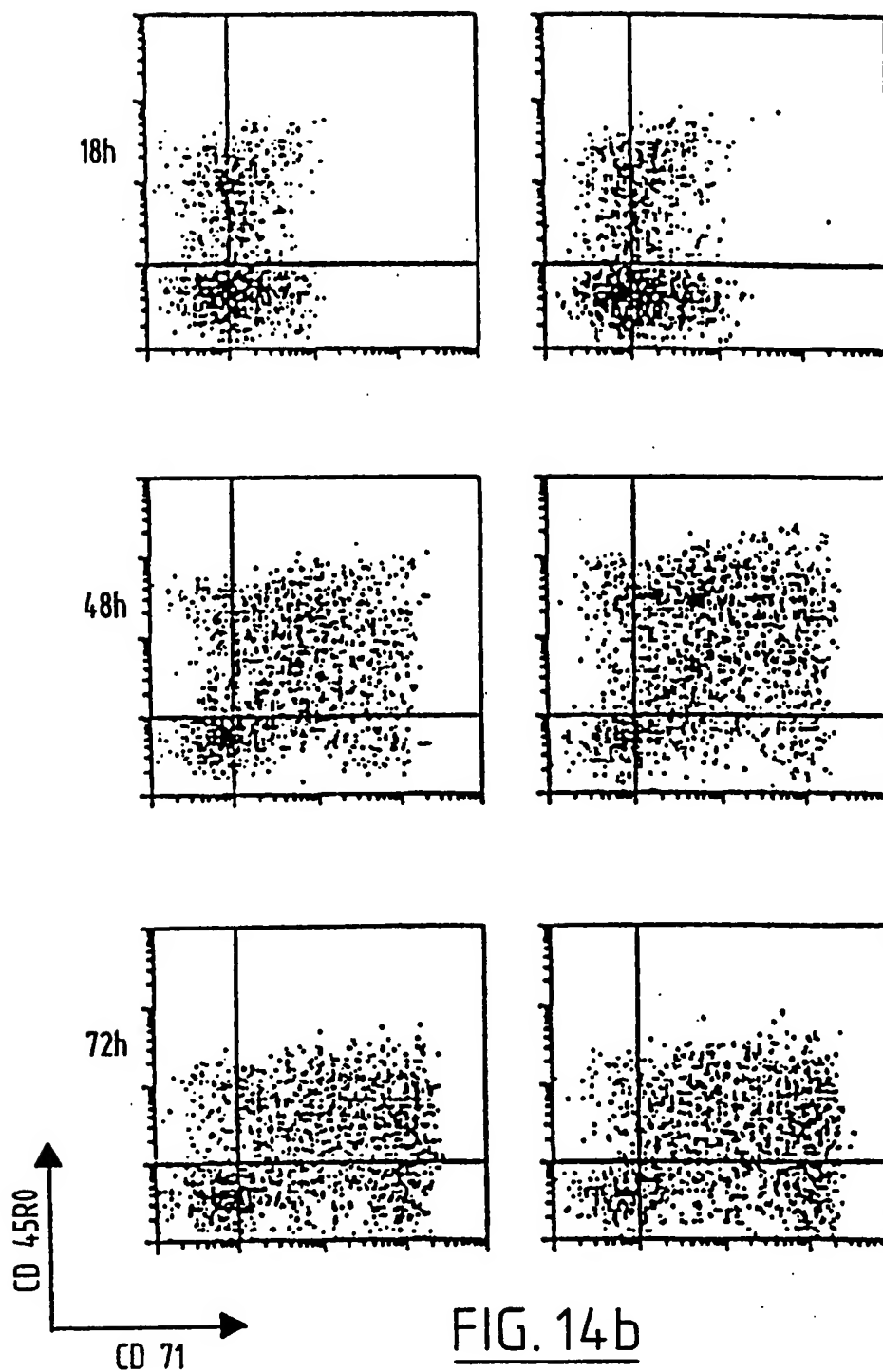
CD 45R0  
CD 71

FIG. 14b

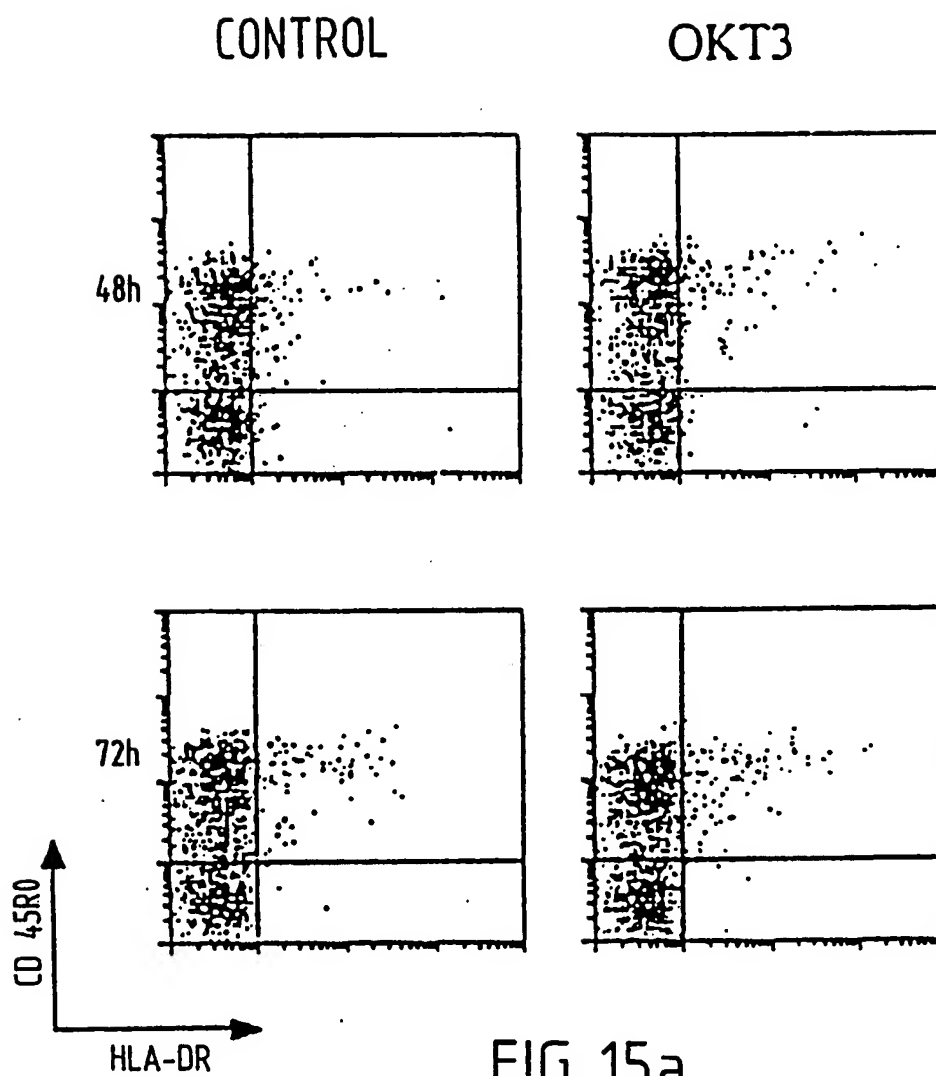
25/30

OKT3/Ta1

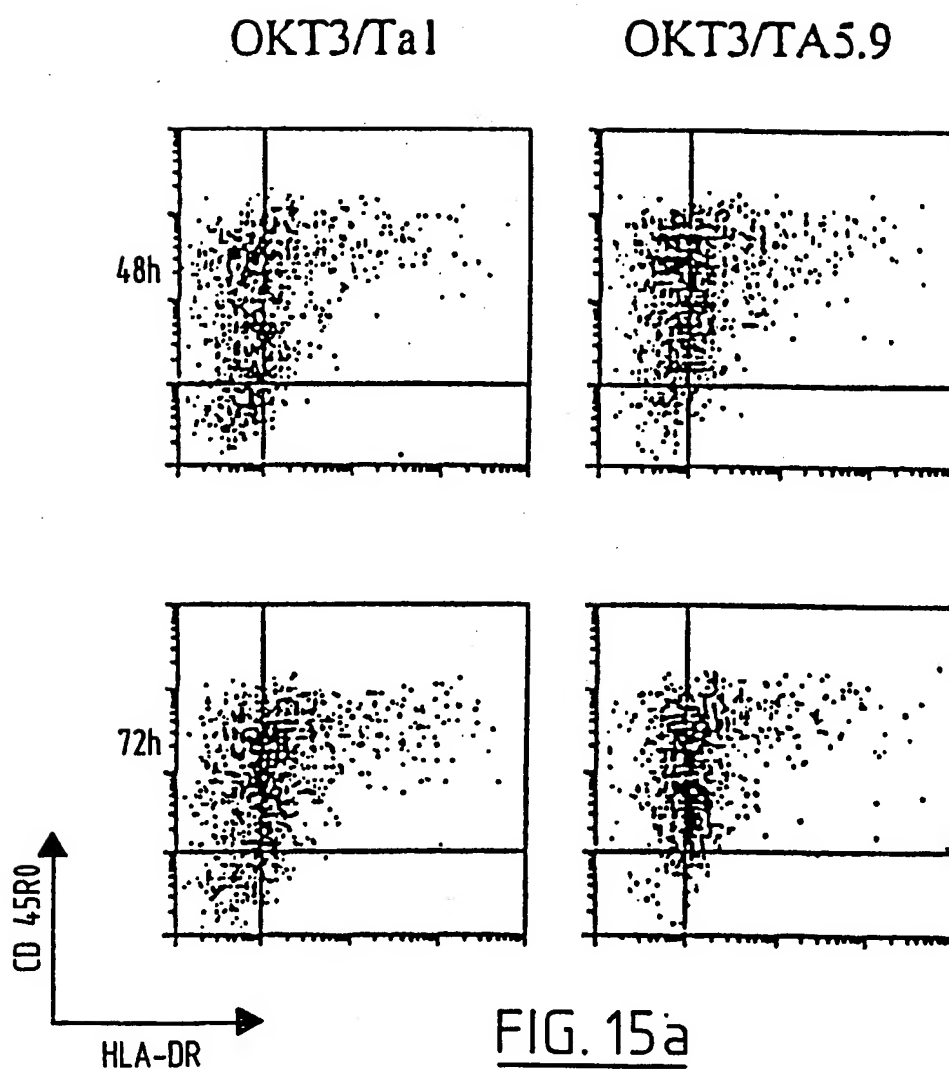
OKT3/TA5.9



26/30

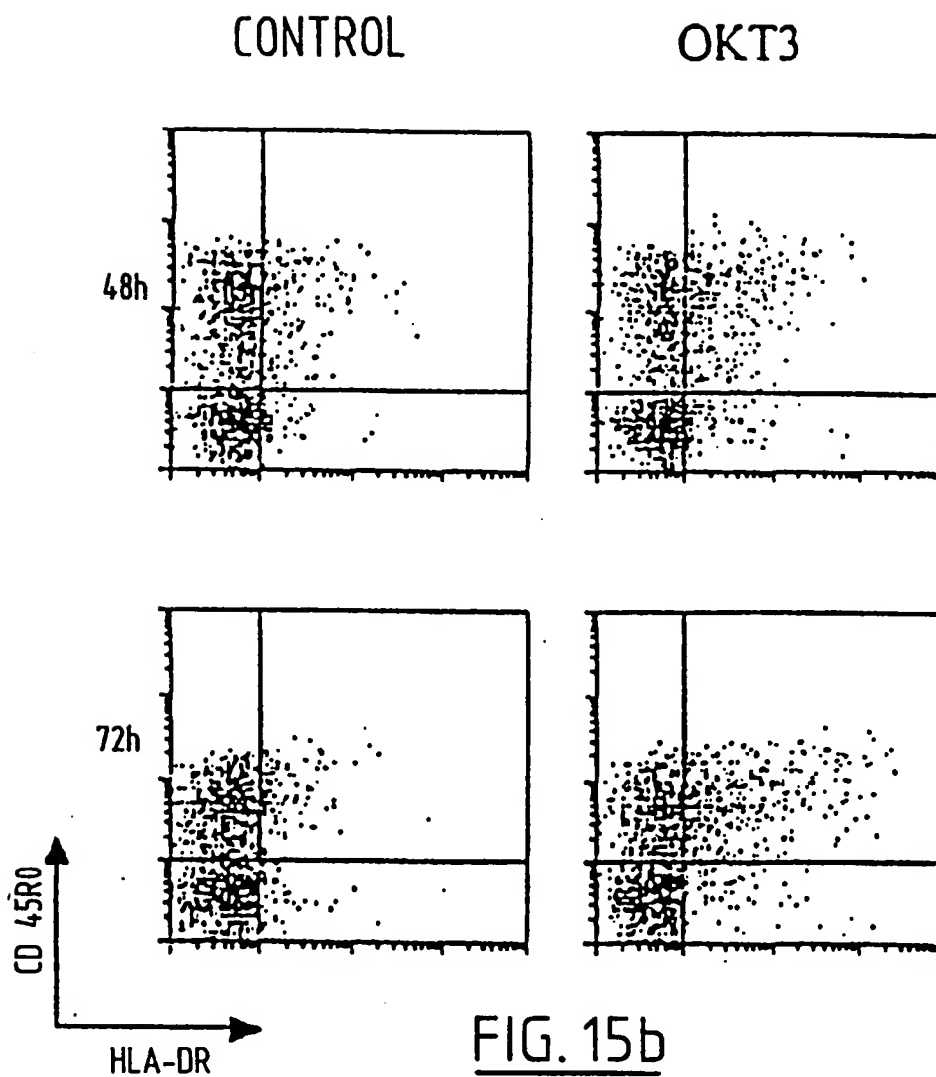


27/30

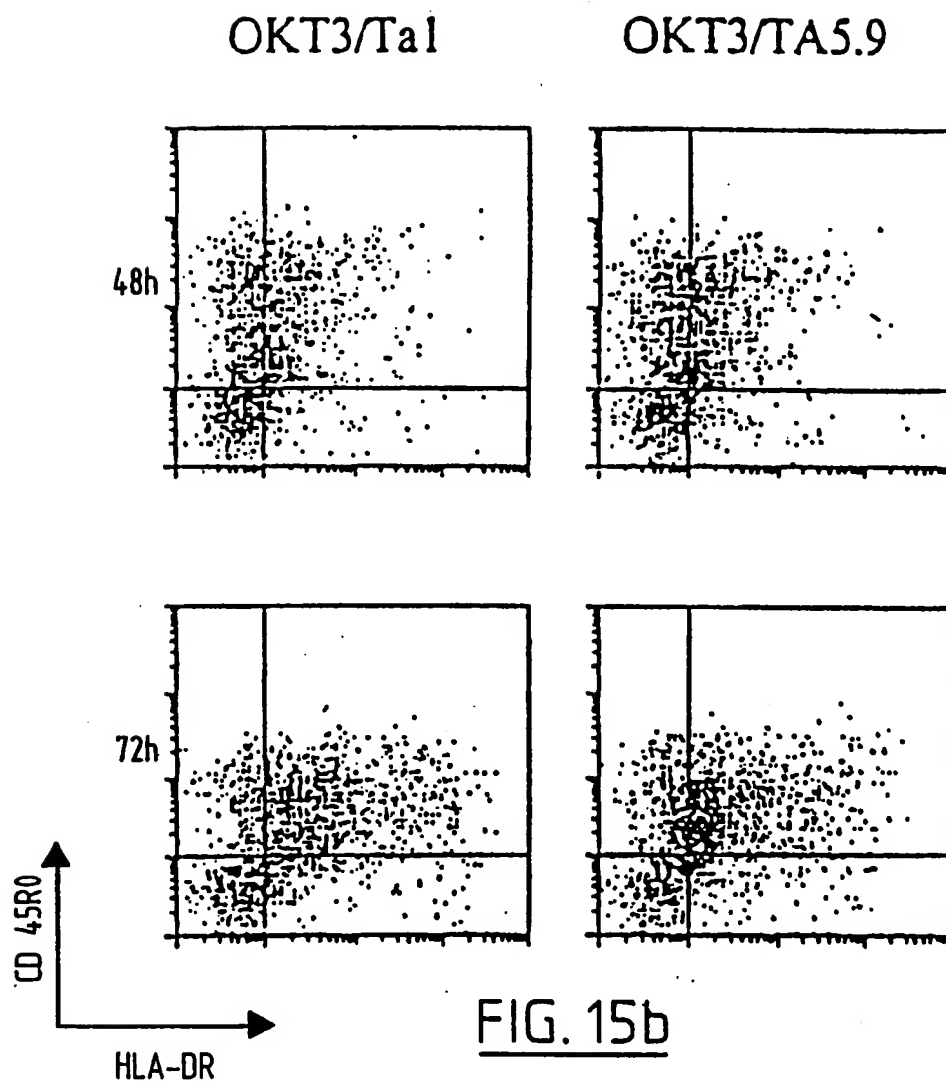




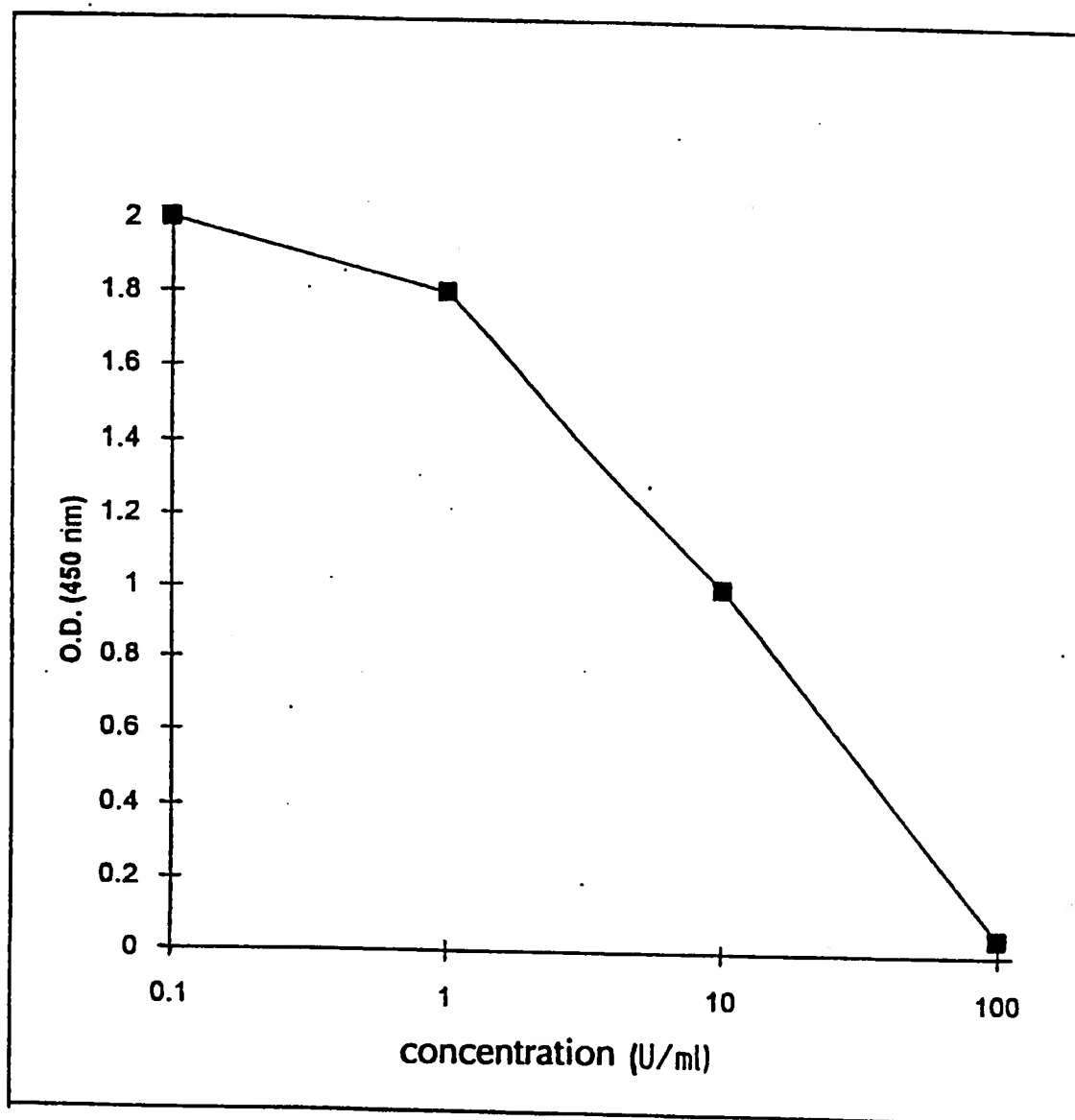
28/30



29/30



30/30

FIG. 16

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 94/03632

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K16/40 C12N9/78 A61K39/395 A61K38/50 A61K51/08  
G01N33/573 G01N33/574 G01N33/577 C12N5/20

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TISSUE ANTIGENS, vol.42, no.4, 1993, COPENHAGEN, DENMARK page 242 I. DE MEESTER ET AL. 'Down-regulation of CD26 surface expression by anti-TA5.9 monoclonal antibody.' zie samenvatting AA011 ---	1-5,7,8, 10-16
X	IMMUNOBIOLOGY, vol.188, no.1-2, June 1993, STUTTGART, GERMANY pages 145 - 158 I. DE MEESTER ET AL. 'Antibody binding profile of purified and cell-bound CD26. Designation of BT5/9 and TA5.9 to the CD26 cluster.' cited in the application see the whole document ---	1-5,7,8, 10-16

--/--

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

6 January 1995

Date of mailing of the international search report

13. 01. 95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 94/03632

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THE JOURNAL OF EXPERIMENTAL MEDICINE, vol.177, no.4, 1 April 1993, NEW YORK NY, USA pages 1135 - 1143 M. MORRISON ET AL. 'A marker for neoplastic progression of human melanocytes is a cell surface ectopeptidase.' cited in the application see figures 3,4 see abstract see page 1139, left column, line 7 - right column, line 7</p> <p>---</p>	1,2,4,5, 7,8, 10-15
A	<p>THE SCANDINAVIAN JOURNAL OF IMMUNOLOGY, vol.31, no.4, April 1990, OXFORD, GB pages 429 - 435 A. ULMER ET AL. 'CD26 antigen is a surface dipeptidylpeptidase IV (DPPIV) as characterized by monoclonal antibodies clone TII-19-4-7 and 4EL1C7.' cited in the application see abstract</p> <p>---</p>	1-5,7,8, 12
A	<p>MOLECULAR IMMUNOLOGY, vol.29, no.2, February 1992, OXFORD, GB pages 183 - 192 Y. TORIMOTO ET AL. 'Biochemical characterization of CD26 (dipeptidyl peptidase IV): functional comparison of distinct epitopes recognized by various anti-CD26 monoclonal antibodies.' see abstract</p> <p>---</p>	1-5,7,8, 10,12
P,X	<p>EUROPEAN JOURNAL OF IMMUNOLOGY, vol.24, no.3, March 1994, WEINHEIM, GERMANY pages 566 - 570 I. DE MEESTER ET AL. 'Binding of adenosine deaminase to the lymphocyte surface via CD26.' see the whole document</p> <p>-----</p>	1-5,7,8, 10-16